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Substrate utilization in skeletal muscle and adipose tissue

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SUBSTRATE UTILIZATION IN SKELETAL MUSCLE AND ADIPOSE TISSUE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Obesity and Type 2 diabetes are associated with high fat diet (HFD), reduced mitochondrial mass and function and insulin resistance as characterized by glucose disposal and relative to body fatness.

We hypothesized that (a) HFD affects expression of genes involved in mitochondrial biogenesis and function, (b) carbohydrate metabolism and storage is under transcriptional control and (c) both overall fatness and characteristics of adipose tissue influence the interplay between free fatty acids (FFAs) and insulin-stimulated glucose disposal. To test hypotheses "a" and "b", we fed 10 insulin-sensitive males an isoenergetic HFD for 3 days with muscle biopsies before and after intervention. Oligonucleotide microarrays revealed 370 genes differentially regulated in response to HFD (Bonferonni adjusted $p < 0.001$). Expression of six genes involved in oxidative phosphorylation decreased. PGC1α and PGC1ß mRNAs decreased by ~22%. Seven genes in the carbohydrate metabolism pathway changed in response to HFD, and three genes confirmed by qRT-PCR: PFKFB3, PDK4 and GYS1. In a separate experiment, C57Bl/6J mice were fed HFD for three weeks and the same OXPHOS and PGC1 mRNAs decreased by ~90%, Cytochrome C and PGC1α protein by ~40%, while the same glucose metabolism genes changed by ~70%. These results suggest a mechanism whereby HFD downregulates genes necessary for oxidative phosphorylation and mitochondrial biogenesis, as well as glucose utilization and storage. These changes mimic those observed in diabetes and insulin resistance.

To test hypothesis "c", we measured changes in respiratory quotient (ΔRQ; metabolic flexibility) before and during euglycemic-hyperinsulinemic clamps in healthy

young males. Anthropometric, laboratory measurements, fat biopsies and fat cell size (FCS) were measured after overnight fast. Adipose tissue gene expression (qRT-PCR) was measured. Metabolic inflexibility (lower ΔRQ) was associated with higher body fat, larger FCS and higher insulin-suppressed FFAs. ΔRQ was not related to fasting FFAs, but lower ΔRQ was associated with lower serum adiponectin levels. Higher adipose tissue inflammatory gene expression was associated with higher insulin-suppressed FFAs and lower ΔRQ. These results indicate fatness, adipocyte hypertrophy, blunted insulin suppression of FFAs, decreased adiponectin levels and inflammation, are associated with decreased insulin-stimulated glucose uptake and oxidation, an important component of metabolic inflexibility.

CHAPTER 1: INTRODUCTION

1.1 Obesity and Diabetes: A Pandemic

An obesity pandemic threatens to overwhelm health systems around the globe with illnesses such as diabetes and heart disease, and this pandemic of obesity is as big of a threat to the world's population as global warming and bird flu. The World Health Organization (WHO) says more than 1 billion adults are overweight and 300 million of them are obese; furthermore, to date more overweight people exist in the world than the undernourished, who number about 600 million (1).

Obesity is a major cause of premature mortality with severe economic consequences. A recent study estimated the annual cost of obesity in the United States to be $93 billion per year, or 9.1% of all healthcare dollars (2). A recent survey by the United States Centers for Disease Control indicates that 66% of the U.S. population are overweight, with 32.3% having a body mass index (BMI) of more than 30 kg/m^2 (3).

The current worldwide pandemic of obesity and its major complications, namely Type 2 diabetes and cardiovascular disease (CVD), is well documented. "Thrifty" metabolic traits have evolved in the setting of decreased famine to give rise to the obesity pandemic. These thrifty traits, specifically a decreased capacity for dietary thermogenesis and an increased resistance to insulin-mediated glucose uptake in skeletal muscle, would historically prolong survival during famine but now predispose individuals to obesity and diabetes in the face of abundance (4-6).

Body-fat distribution is an important factor in determining high-risk obesity. People with abdominal obesity are at greater risk for heart disease, diabetes, hypertension and hyperlipidemia compared to people with a more gluteal fat distribution (7). The reason for the association between central abdominal obesity and metabolic syndrome is not clear, but one leading concept

maintains that visceral adipose tissue has a higher rate of lipolysis, resulting in elevated portal non-esterified fatty acids (NEFAs) increasing hepatic very-low-density lipoprotein (VLDL) production, hepatic glucose output (HGO) and impairing peripheral insulin sensitivity (8).

Considerable evidence exists in the literature for increased inflammation preceding the onset of obesity-related metabolic disorders. Many studies have described correlations between vascular disease and elevated levels of interleukin-6, C-reactive protein and chemokines such as macrophage chemoattractant protein-1 (MCP-1) all of which promote a proinflammatory state (9). Although the origin of this proinflammatory state is not clear, it is important to note the commonalities among the development of obesity and certain features of the metabolic syndrome, such as insulin resistance and vascular disease. All of these suggest that adipose tissue itself may be an important source of proinflammatory cytokines.

The role of adipose tissue in metabolic syndrome and inflammation has continued to evolve with the discoveries of numerous secretory proteins from adipocytes, commonly referred to as adipokines. These adipokines are important determinants of insulin resistance, either through a hormonal effect in the circulation, or through local effects on the adipocytes themselves. Tumor necrosis factor-α (TNFα) expression by adipose tissue of obese rodents and humans was discovered in the mid-1990s (10; 11). Numerous other proteins are secreted by adipocytes, showing adipose tissue is an active metabolic and endocrine tissue. Many of the numerous adipokines expressed by adipose tissue have been implicated in the pathogenesis of metabolic syndrome. For example, plasma levels of interleukin-6 and adipose-secreted TNFα are associated with obesity, as well as with insulin resistance not related to obesity (12). Several mechanisms exist for the actions of TNFα on insulin resistance, including an inhibition of insulin-receptor signaling (13), along with an autocrine effect on adipose tissue causing a

stimulation of lipolysis and elevation of plasma NEFAs (14). Consistent with this model, TNFα-knockout mice do not become insulin resistant with diet-induced obesity (15).

Another important secretory protein of the adipocytes is adiponectin. Unlike other adipokines, adiponectin acts as an anti-diabetic hormone (16). Adiponectin circulates as complex multimeric forms (17), and two receptors for adiponectin have been described (18). Adiponectin-knockout mice develop marked insulin resistance while consuming high-fat diets, and this insulin resistance is improved following adiponectin supplementation (19). In humans, blood levels of adiponectin are decreased under conditions of obesity, insulin resistance, Type 2 diabetes and coronary disease (20; 21).

Recent studies, however, have suggested that the cell producing many of the adipose tissue cytokines is not the adipocyte, but instead is the macrophage that infiltrates adipose tissue during obesity (22; 23). Once activated, macrophages secrete a plethora of cytokines such as TNFα, interleukin-6 and interleukin-1 (24), and the adipose tissue resident macrophages were found to be responsible for the expression of most of the tissue TNFα and interleukin-6 (25; 26). In human studies, there is a correlation between adipose macrophage markers and BMI (22), and an even stronger relationship with insulin resistance. The expression of TNFα and interleukin-6 correlate strongly with the expression of macrophage markers from adipose tissue (27). These adipose-tissue macrophages elucidate our understanding of inflammation, obesity and the metabolic syndrome. Recent data suggest that progressive obesity leads to adipose-tissue macrophage infiltration, at least in some subjects, with consequent over-expression of inflammatory cytokines, metabolic syndrome and a state of heightened inflammation (24; 28).

The prevalence of obesity is increasing at an alarming rate, and the obesity pandemic is driving the pandemic of Type 2 diabetes. High-risk obesity is characterized by abdominal

obesity with abnormal glucose and lipid metabolism, and a state of increased inflammation. As body weight increases, lipid accumulation occurs in adipose tissue, as well as in other organs, the latter effect commonly referred to as 'lipotoxicity'. Lipotoxicity in liver, muscle and the pancreatic islets may account for many of the features of the metabolic syndrome. Consequently, it may be a culmination of the effects of all four tissues (pancreatic β-cells, liver, adipose tissue and skeletal muscle) that leads to the state of insulin resistance and ultimately overt Type 2 diabetes.

Adipose tissue is the primary organ of lipid storage; however, in the case of obesity, lipid is deposited into other non-adipose organs, including liver, skeletal muscle, β-cells and cardiac tissue, leading to 'ectopic fat'. The ectopic fat deposited in the liver leads to the commonly observed hepatic steatosis, also known as non-alcoholic steatohepatitis or "fatty liver". Together, the lipotoxicity in these organs leads to insulin resistance, impaired insulin secretion and eventually Type 2 diabetes. Because the majority of glucose disposal occurs in muscle (29), this tissue is extremely important in understanding the mechanisms underlying peripheral insulin resistance. Insulin-resistant subjects demonstrate increased intramyocellular lipid, a decreased proportion of the oxidative type I and type IIA muscle fibers, decreased oxidative capacity of each class of muscle fiber and decreased capillary density (30; 31). Recent studies that compared insulin-resistant and insulin-sensitive subjects have suggested the presence of a reduced mitochondrial lipid oxidation as an early defect (32; 33). Improvement in the intramyocellular lipid and muscle lipid oxidative capacity accompanies improvement in insulin resistance following weight loss (34), and in response to treatment of insulin-resistant subjects with pioglitazone, which causes a shift in lipid from ectopic sites to subcutaneous adipose tissue (35).

A reduction, therefore, in intramyocellular lipid flux or an increased sequestation of lipid in adipose tissue may be an attractive target for future drug therapies.

Indeed, muscle impairments in insulin-mediated glucose transport, glycogen synthesis and glucose oxidative pathways are the central features of individuals at high risk for Type 2 diabetes, such as the offspring of Type 2 diabetics (36; 37). As these population groups also present with elevated plasma concentrations of fatty acids and amino acids, these substrates (by interfering with the uptake and metabolism of glucose in skeletal muscle) are thought to play a central role in the onset of insulin resistance (36; 38-40). Furthermore, there is also evidence that as chronic hyperglycemia develops, the oversupply of glucose could also, by inhibiting fatty acid oxidation, lead to the accumulation of intramyocellular lipid that further inhibits glucose uptake and subsequent metabolism and hence exacerbates the state of insulin resistance (e.g. the 'reverse Randle Cycle') (41-43).

The complications of obesity affect all specialties of medicine, and the effective treatment of obesity will be one of the major challenges for the 21st century. Until effective medical therapy becomes available, it is important to identify patients with evidence of the metabolic syndrome and other features of high-risk obesity and to treat the diabetes, hyperlipidemia, inflammation and other associated co-morbidities effectively.

The treatment of obesity requires the identification of the high-risk patient, the institution of lifestyle measures with a long-term outlook and an avoidance of extreme fad diets. Current research will likely lead to improved medications in the future.

1.2 Substrate Utilization

Substrates compete for respiration. Beginning in the 1930s Krebs (44) showed competition between amino acids and glucose in kidney slices. Subsequent to Krebs, Waters et

al demonstrated a competition between 3-hydroxybutyrate and glucose in dog heart lung preparations (45). However, general acceptance of the concept came in 1963 with Sir Randle's demonstration that lipid fuels inhibit glucose oxidation in rat diaphragm and perfused heart (46; 47). This lead to Randle et al (48; 49) proposing a 'glucose-fatty acid cycle' as an important set of mechanisms by which carbohydrate and fat metabolism interact. As recently as 1998, Randle revisited the essential components of this cycle (50): (1) the relationship between glucose and fatty acid oxidation is reciprocal and not dependent, (2) *in vivo*, the oxidation of lipid fuels (fatty acids and ketone bodies) released into the circulation (e.g., in starvation or diabetes) may inhibit the catabolism of glucose in muscle and (3) *in vitro*, the oxidation of fatty acids released from muscle triacylglycerol or intramyocellular triglycerides also inhibits intracellular glucose metabolism.

Frayn reevaluated the 'glucose-fatty acid cycle' from a physiological perspective and pointed out that it is appropriately termed a 'cycle' because it describes a series of events that interlink carbohydrate and fat metabolism (51). Elevated glucose concentrations (as in the post-prandial state after a meal) stimulate insulin secretion, which then suppresses free fatty acid (FFA) release from adipose tissue. This eliminates competition for substrate utilization in skeletal muscle, so that glucose utilization may be stimulated by insulin, unaffected by high concentrations of fatty acids. Conversely, when plasma FFAs are high, which is usually because glucose and insulin concentrations are low (as in the post-absorptive state), fatty acids then become the major fuel for skeletal muscle. According to Frayn, "This makes perfect physiological sense in terms of blood glucose homeostasis. The 'coarse' control of the reciprocal utilization of glucose and FFA in the body is brought about through insulin secretion: fine-tuning is provided in skeletal muscle" (51).

The Randle (glucose-fatty acid) Cycle embodies the concept of direct competition between substrates for mitochondrial oxidation. A plethora of mechanisms have been put forward concerning the biochemical regulation of fuel homeostasis in skeletal muscle (40; 41). Interestingly, the oversupply of each of the three main fuel substrates (fatty acids, amino acids, glucose) converges towards the accumulation of triglycerides. This can occur when circulating free fatty acids (FFAs) exceed FFA oxidation, as well as when amino acids from protein or glucose from carbohydrate exceed their oxidation; malonyl-CoA is formed in each case driving lipid synthesis (52). Malonyl-CoA inhibits carnitine palmitoyltransferase-1 (CPT-1), which reduces entry of long-chain fatty acyl-CoAs into mitochondrial fatty acid oxidation. The consequence of this excess unoxidized lipid leads to an effect known as 'lipotoxicity', which can lead to the development of intramuscular lipid droplets. Intramuscular lipid impairs insulin signaling, which is necessary for insulin-stimulated glucose uptake and consequently glucose metabolism. Lipotoxicity can also lead to ceramide synthesis and lipid peroxidation via nonoxidative metabolic pathways, leading to cell dysfunction and death through apoptosis (53).

The term 'lipotoxicity' indicates toxicity that may result from lipid overloading induced by delivery exceeding oxidation of circulating FFA and local release of FFAs from triglyceride stores by lipoprotein lipase (LPL), but also from that induced by glucose overloading (gluco-lipotoxicity) and protein overloading (or proteo-lipotoxicity). These models of lipotoxicity explain how nutrient supply exceeding oxidation may lead to insulin resistance in skeletal muscle. In addition, fuel oxidation matches increased fuel supply vis-à-vis cellular nutrient sensors and signaling systems, and contributes to blood glucose homeostasis and protection against muscle lipotoxicity. Tthe interactions between glucose and lipid metabolism in skeletal muscle in relation to blood glucose homeostasis, and subsequently these interactions with respect

to thermogenic mechanism of substrate cycling could offer protection against skeletal muscle lipotoxicity.

The inhibitory effect of elevated lipid fuels (fatty acids and ketone bodies) on glucose disposal in skeletal muscle has been established by numerous studies conducted both *in vivo* and *in vitro*. In humans, it has been shown that under *in vivo* conditions when fatty acid concentrations are elevated (e.g., in response to lipid infusion) whole body, as well as skeletal muscle glucose utilization, is impaired (54-56). The plethora of mechanisms put forward to explain how fatty acids limit insulin-stimulated glucose utilization fall into two main categories. Both categories were first proposed by Randle following the original studies which formed the basis of the 'glucose-fatty acid cycle': (1) fatty acid-induced desensitization of insulin-mediated glucose transport and (2) inhibitory effects of fatty acid oxidation (46-49). According to Randle, an increase in lipid oxidation will decrease glucose oxidation by suppression of the mitochondrial pyruvate dehydrogenase (PDH) complex, with the subsequent reduction of glycolytic flux, which results in an increase in glucose-6-phosphate, inhibition of hexokinase activity and ultimately decreased glucose uptake. As a result of this, nonoxidative metabolism (storage) of glucose should decrease. A variety of model systems have largely confirmed the validity of Randle's mechanism, at least in terms of the acute effects of fatty acids on muscle glucose metabolism and storage (50; 57).

In the late 1970s McGarry et al elucidated the reciprocal nature of interactions between glucose and lipid metabolism by demonstrating that high glucose (and insulin) concentrations can suppress hepatic fatty acid oxidation through malonyl-CoA inhibition of CPT-1, a key rate-limiting enzyme that controls the entry of fatty acids into the mitochondrial fatty acid oxidation system (58; 59). This mechanism by which glucose regulates fatty acid oxidation is

complementary to the mechanism described by Randle et al and is often referred to as the 'reverse glucose-fatty acid cycle'. Malonyl-CoA inhibition of fatty acid oxidation contributes to the switch to pyruvate oxidation; however, inhibition of acetyl-CoA carboxylase (ACC), which produces malonyl-CoA from acetyl-CoA, enables fatty acid oxidation at the expense of glucose oxidation. This 'reverse glucose-fatty acid cycle' can be viewed as further 'fine-tuning' of the balance between glucose and fatty acid metabolism, and it adds weight to the bi-directionality of the cycle.

Recently, a new twist and important question was posed regarding glucose inhibition of fatty acid oxidation, namely: "How can one substrate have opposing roles?" Mitochondrial fatty acid oxidation is inhibited by an elevation in malonyl-CoA, an intermediate substrate (formed from acetyl-CoA by ACC) that is also a precursor for *de novo* fatty acid synthesis--hence, the paradox (60). The role of malonyl-CoA as a precursor of lipid synthesis and as an inhibitor of lipid oxidation was eventually reconciled with the discovery of two isoforms of ACC (61; 62). ACC1 is expressed predominantly in lipogenic tissues that synthesize large amounts of fatty acids (liver and adipose tissue) and ACC2 is expressed in non-lipogenic tissues such as skeletal muscle and cardiac muscle. ACC1 is thought to reside in the cytoplasm, where it synthesizes the pool of malonyl-CoA that is used for *de novo* lipogenesis; whereas, ACC2 is thought to control the pool of malonyl-CoA that regulates fatty acid oxidation.

In skeletal muscle, the suppressive effect of elevated glucose on fatty acid oxidation occurs via malonyl-CoA (synthesized by ACC2) inhibition of CPT-1, but the question of whether a cytoplasmic pool of malonyl-CoA might be used for fatty acid synthesis has not been raised. Historically, skeletal muscle is not an organ where *de novo* lipogenesis occurs. Whenever the expression or activity of a rate-limiting enzyme for *de novo* lipogenesis (e.g., fatty

acid synthase) was reported in skeletal muscle, this was just thought to be adipocyte contamination rather than *de novo* lipogenesis occurring in myocytes (52; 63). As a consequence, the role of skeletal muscle substrate metabolism in blood glucose homeostasis has been viewed from the relationship between glucose and lipid metabolism. Recent evidence, however, suggests that skeletal muscle has a more active role. *De novo* lipogenesis can occur in muscle cells and be modulated by factors that influence the body's nutritional state; therefore, glucose and lipid metabolism may actually depend on, rather than respond to, each other for these two fuel substrates in skeletal muscle. This is in contrast to Randle's revisit of the 'glucose-fatty acid cycle' in 1998 (50).

It has been proposed that there is an interdependency between glucose, lipids and thermogenesis. Acetyl-CoA produced from glucose and fatty acid oxidation may overload the Krebs cycle. This, in turn, results in excess mitochondrial citrate, which activates ACC2 and also provides substrate (in the form of acetyl-CoA) to ACC2 for the synthesis of malonyl-CoA. Malonyl-CoA serves as the main substrate for fatty acid synthase (FAS), which produces a new pool of fatty acids. On the other side of the equation, glucose plays a central role in this cycle as a source of acetyl-CoA, Krebs cycle intermediates and NADPH molecules, which are required for fatty acid synthesis. Glucose might also function as a stimulator of *de novo* lipogenesis, based on recent evidence in rat muscle satellite cells that glucose stimulates expression of genes encoding glycolytic and lipogenic enzymes, leading to an increased lipogenic flux (64).

Skeletal muscle adapts to three different physiological conditions by increasing fat oxidation: (1) reduced energy intake during fasting, (2) increased energy expenditure during exercise (65) and (3) in obesity (66-68). The transition to fat oxidation for energy spares glucose metabolism during fasting and delays muscle glycogen metabolism during exercise. Almost 50

years ago, it was reported that even in lean, healthy volunteers after just an overnight fast, skeletal muscle chiefly relies upon fat oxidation for fuel supply (69). Recently, it has been proposed that inability to increase reliance upon fat oxidation is related to the pathogenesis of insulin resistance in skeletal muscle and perhaps to the pathogenesis of obesity (70; 71). To this end, Ukropcova et al examined the capacity for fat oxidation in skeletal muscle by using primary human skeletal muscle cells obtained from biopsy of the vastus lateralis. They found that fatty acid oxidation was increased in cells from subjects with higher insulin sensitivity, leanness and aerobic fitness (72).

The physiologic purpose of alterations in fuel selection is to channel energy to and from the appropriate storage compartments under all circumstances that may confront the organism. An obligate need exists to regulate glycogen within a relatively narrow window. Studies conducted in subjects with Type 2 diabetes and in healthy subjects to determine the fate of glucose after it is taken up by muscle cells demonstrate that muscle glycogen synthesis, along with glycolysis, is an important pathway in overall skeletal muscle glucose metabolism (76). Muscle cells adjust the fuels they oxidize in order to match substrate supply, signals from the endocrine and neural systems and the ATP required for contraction. The supply of substrate to muscle tissue depends upon several factors including the dietary macronutrient content, the storage of nutrients in liver and fat, capillary recruitment and transport, and in the case of triglycerides, the release of free fatty acids by the enzymatic activity of lipoprotein lipase (LPL) at the surface of the cell. The adjustment of substrate oxidation to the local nutrient concentrations occurs through at least three mechanisms: minute-to-minute changes in the activity of enzymes that direct carbohydrate and fat into oxidation or storage (86), the activation of signaling pathways such as PKC (98) and NFκB (190) and through long-term regulatory systems that

involve changes in gene transcription and hence the cellular machinery driving the first two processes. The ability of insulin to regulate glucose homeostasis, i.e. insulin sensitivity, is closely related to these regulatory pathways, justifying further attempts to unravel the details of these regulatory systems. Strong experimental evidence exists for each of these pathways, particularly for the regulation of substrate utilization and insulin sensitivity through enzyme activity (87) and signaling pathways (98).

Skeletal muscle plays a key role in determining systemic insulin sensitivity. Upon insulin stimulation the majority of glucose metabolism occurs in the skeletal muscle. Impaired glucose metabolism in muscle constitutes peripheral insulin resistance and is seen in Type 2 diabetes and obesity. Is the impairment of skeletal muscle to increase fat oxidation under appropriate conditions also related to insulin resistance? Insulin resistance in muscle can be induced by elevated plasma fatty acids levels. High FFAs are also associated with increased fat oxidation (54; 57). Maintenance of fasting levels of plasma fatty acids through lipid infusions significantly lowers insulin-stimulated glucose uptake by skeletal muscle, impairs insulin suppression of lipid oxidation and blunts stimulation of glucose oxidation in muscle (56). These experimental conditions, which induce insulin resistance in skeletal muscle by elevating circulating free fatty acids, mimic those found in obesity and Type 2 diabetes (73; 74). These observations complicate the evidence that insulin-resistant skeletal muscle can also demonstrate reduced efficiency of fat oxidation during fasting despite high levels of plasma free fatty acids, which are almost invariably present in obesity and Type 2 diabetes, as well as high very low density lipoprotein (VLDL) triglycerides and intermediate density lipoprotein (IDL) triglycerides.

Kelley et al showed a high reliance of leg muscle on fat oxidation (lower RQ) during fasting conditions. In these same individuals, insulin infusion suppressed fat oxidation and

shifted to a high reliance on glucose oxidation (higher RQ) (75); therefore, metabolically healthy skeletal muscle is characterized by the ability to switch easily between glucose and fat oxidation in response to homeostatic signals and substrate availability. The skeletal muscle of individuals with Type 2 diabetes (T2D) and obesity demonstrates decreased metabolic flexibility. Even though RQ values during fasting are higher in T2D and obesity, stimulation of glucose oxidation in response to insulin is blunted (76; 77). This phenomenon of responding inefficiently both to the fasting stimulus to enhance fat oxidation and to the insulin stimulus to turn on glucose oxidation has been termed "metabolic inflexibility". Ukropcova et al examined metabolic flexibility in their cultured myocytes (72) and found a match between the cellular characteristics and the whole body "metabolic flexibility" of the cellular donor. Furthermore, Goodpaster et al found that moderate weight loss, combined with an improvement in aerobic capacity, can restore metabolic flexibility and improve insulin-stimulated glucose disposal in overweight and obese insulin-resistant individuals (78). Interestingly, however, moderate weight loss without a change in aerobic capacity does not improve fat oxidation under fasting conditions, rather it improves suppression of fat oxidation during insulin-stimulated conditions (79; 80).

Metabolic flexibility involves: (1) insulin suppression of fat oxidation with stimulation of glucose oxidation, and (2) stimulation of fat oxidation during fasting. In the study by Ukropcova et al, glucose had a varying effect on the suppression of fat oxidation in the primary human myocytes. Resistance to suppression of fat oxidation by hyperglycemia was inversely related to insulin sensitivity, percent body fat and aerobic capacity (72).

Another study by Henry et al found that characteristics of insulin resistance in skeletal muscle are retained in myocyte culture (81), and it has been reported that muscle obtained from Type 2 diabetics has reduced capacity for fat oxidation in culture (82). Additionally, the results

reported by Ukropcova et al demonstrate metabolic flexibility (the transition between fat and glucose oxidation) is a cellular characteristic that is retained *in vitro*. It is not clear what the cellular characteristics of myocytes determine metabolic flexibility, but Ukropcova et al did show that mitochondria play a critical role. Competition between glucose and fat oxidation can occur at several locations in the mitochondria. One location is the mitochondrial outer membrane where pyruvate dehydrogenase and carnitine palmitoyl transferase complexes compete for acetyl-CoA. Competition may also occur in the mitochondrial matrix between β-oxidation (fatty acid oxidation) and the tricarboxylic acid (TCA) cycle, as well as in the delivery of $FADH_2$ and NADH molecules to the electron transport chain (ETC) for oxidative phosphorylation in the inner mitochondrial membrane.

Recently, mitochondria have become a hot topic in the investigation of the pathogenesis of muscle insulin resistance (83-87). In addition to clinical investigations, suitable animal models for further study are also being developed. For example, rats selectively bred for low oxidative enzyme activity in skeletal muscle develop a metabolic syndrome phenotype (88). Also, in studies that examined the physiological phenotype of metabolic flexibility, muscle biopsy samples have shown reduced oxidative enzyme activity (33; 80; 89).

In the future it will be important to examine the links between substrate utilization and mitochondrial metabolism and incorporate the findings into the broader spectrum of metabolic pathways within insulin-sensitive tissues, such as liver, adipose tissue and skeletal muscle. Understanding metabolic adaptation to our nutritional environment is important to the future of obesity and diabetes research.

1.3 The ADAPT Study Design

The ADAPT study began in 2000 and completed in 2003. In the past several years a "thrifty phenotype" has been identified and characterized in lean men by Smith et al as an inability to adapt rapidly to a high fat diet by increasing fatty acid oxidation. It is associated with a low maximal VO_2 during exercise and a high fasting insulin. We hypothesized that individuals with the "thrifty phenotype" are at higher risk for becoming obese, and that exercise may be effective in overcoming this problem. The latter hypothesis was supported as treadmill exercise increased the rate of adaptation to a high fat diet (90).

Several questions remained to be answered regarding this "thrifty phenotype". First, given the large interindividual differences in fatty acid oxidation, how could we identify those individuals at the highest risk? What would be the distinguishing biochemical, endocrine and environmental characteristics of individuals that store fat when exposed to high fat diets? This was important because if these individuals could be easily identified, then dietary and other interventions could be targeted to this "at-risk" population.

Second, what would be different about the individual with the "thrifty phenotype"? What would be the cellular pathways dysregulated in the skeletal muscle of these individuals? If the defect was intrinsic, i.e. a diminished ability to conserve glucose and oxidize fat in skeletal muscle or alternately, was the phenotype due to environmental, and dietary factors such as inactivity and energy excess?

To answer these questions we planned a three-year project that aimed to: (1) characterize the biochemical, endocrine, anthropometric and environmental characteristics of individuals with the "thrifty phenotype", (2) identify the signaling pathways in skeletal muscle that are dysregulated in individuals with the "thrifty phenotype" through mRNA expression profiling and

(3) determine the role of environmental factors such as inactivity and caloric intake versus intrinsic (genetic) factors in the "thrifty phenotype".

The first component of the proposed experiment was to compare adaptation to high fat, versus low carbohydrate diets in men versus women, particularly comparing women with a central versus peripheral fat distribution pattern. Capacity to adapt to high fat diets was measured at energy balance in a whole room calorimeter after feeding a high-fat diet on the preceding three days (see experimental schedule below). In an attempt to sort out the relative role of these factors in the "thrifty phenotype" we measured: socioeconomic status, body composition, family history of diabetes and obesity, fat preference, restraint/disinhibition, core body temperature, habitual physical activity, aerobic capacity, insulin sensitivity, energy related hormones and sympathetic nervous system (SNS) activity (as measured by heart rate variability + plasma and urinary catecholamines). We measured food intake after three days of a high fat diet to determine if the decrease in carbohydrate stores in "thrifty" individuals would increase subsequent food intake.

The second component of the proposed experiment was to compare the biochemical and signaling pathways dysregulated in skeletal muscle and adipose tissue of the individual with the "thrifty phenotype" compared to "fat-burning individuals". Before and after the measurement of their capacity to adapt to high fat diets as described above, individuals with high and low fat oxidative capacity underwent an adipose tissue and skeletal muscle biopsy. These samples were examined for gene expression for several candidate genes from pathways involved in substrate utilization and energy/nutrient sensing

In addition to the candidate gene approach, these samples were analyzed using microarray resources at Pennington Biomedical Research Center. The expression data was

analyzed using cluster analysis, gene shaving and other advanced bioinformatics techniques (91-102). We confirmed differentially expressed mRNAs and proteins using Western blotting and/or enzyme assays.

The third component was to determine if the phenotype of impaired fat oxidation was preserved *in vitro*. In this study, skeletal muscle from "thrifty" and "non-thrifty" individuals was cultured *in vitro* and fat versus carbohydrate oxidation was determined at various concentrations of insulin. We then compared the phenotype *in vitro* with the phenotype *in vivo*. If the phenotype was retained, then we concluded that the defect was genetic. If the phenotype was not retained, then we concluded that the defect lie in the neural or endocrine systems or was environmental. Studies of this type were critical in understanding the role of genetics in the metabolic disorder of insulin resistance syndrome (81).

All volunteers had to complete a two-stage screening study. Screening visit one was for blood sampling (chem 16 with lipids, CBC, UA). Screening visit two was for physical examination and familiarization with the metabolic chamber.

Eligible volunteers were screened and signed a consent document. After enrolling into the study, the volunteers completed the baseline measures. After consuming the control diet for one day, they were admitted to the metabolic unit for testing at 6pm. On the next day, baseline blood samples (GLP-1, leptin, ghrelin, resistin, insulin, C-reactive protein (CRP), C-peptide, glucose, NEFA, lipids, study archives) were drawn. After local anesthesia, a muscle and a fat biopsy were performed. The next test was an insulin-glucose clamp to measure insulin sensitivity and metabolic flexibility.

The next four days were spent in the metabolic chamber: one day with the standard diet and three days with the high fat diet. Each day, the volunteers exited the chamber, a butterfly IV

was inserted, they rested in a recumbent condition for 30 minutes and a basal blood sample was collected. After exiting the chamber on the fourth day, volunteers had the last morning blood sample drawn and then consumed the test meal.

Both genders and all races were invited to participate. Women were asked to participate in the follicular phase of the menstrual cycle as determined by menstrual history, and a negative pregnancy test was also recorded prior to participation. Body mass index (BMI) range was $\geq$ 19 and $\leq$ 30. Age range was 18 to 30 years.

Table 1.1 Clinical procedures for the ADAPT study.

procedure	**baseline**	**run-in diet**		**high-fat diet**				
		Su	M	Tu	W	Th	F	Sat
			inpatient	metabolic chamber				
body composition	X							
exercise test	X							
activity monitor	X							
metabolic cart			X	X	X	X	X	
questionnaires			X					
insulin clamp			X					
blood draw				X	X	X	X	
metabolic chamber				X	X	X	X	
core temperature				X	X	X	X	
fasting blood sample				X	X	X	X	X
heart rate variability				X				X
urine collection			X	X	X	X	X	
biopsy			X					X

Smokers were excluded from the study. Volunteers who were unwilling or unable to abstain from alcohol consumption and caffeine consumption prior to testing and laboratory were excluded. Significant renal, hepatic, endocrine, pulmonary, cardiac or hematological diseases were also exclusionary. Women who were pregnant, post-menopausal or taking oral

contraceptives or estrogen replacement therapy were excluded. Other exclusion criteria included: (1) corticosteroid use in the previous two months, (2) chronic use of anti-diabetic, anti-hypertensive or other medications known to affect fat metabolism and (3) weight gain or loss $\geq$ 3kg in the previous six months.

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 Euglycemic-hyperinsulinemic Clamp (EHC)

Insulin sensitivity and metabolic flexibility (change in respiratory quotient (ΔRQ) from fasting to insulin-stimulated) was measured by euglycemic-hyperinsulinemic clamp (103) prior to HFD. After an overnight fast, insulin [80mIU/m^2BSA] was administered intraveneously and glucose infused to maintain plasma glucose at 90mg/dl for 2 hours. The glucose disposal rate (GDR; mg/kgFFM/min), was adjusted for kg of lean body mass.

2.2 Maximal Aerobic Capacity (VO_2 Max)

Maximal oxygen uptake was determined by a progressive treadmill test to exhaustion (104). The volumes of O_2 (VO_2) and CO_2 (VCO_2) were measured continuously using a metabolic cart (V-Max29 Series, SensorMedics, Yorba Linda, California).

2.3 Body Composition (DEXA)

Body fat mass and lean body mass were measured on a Hologic Dual Energy X-Ray Absorptiometer (QDR 2000, Hologic, Inc. Waltham, MA). Visceral fat was measured by CT scanning using a High Speed^{TM} CT scanner under an established protocol (105).

2.4 Indirect Calorimetry

24-hour energy expenditure and respiratory quotient (RQ) were determined in the whole room respiratory calorimeter, prior and during 3 days of isocaloric high fat diet. Energy expenditure was set at 1.4 times the resting metabolic rate and clamped across the 4-day chamber stay. After an overnight fast, fasting and steady state (insulin infusion) respiratory quotient (RQ) were measured for 20 minutes by indirect calorimetry during the EHC using a Deltratrac II indirect calorimeter (DATEX-Ohmeda, Helsinki, Finland). Oxidative and non-oxidative glucose disposal was calculated as described by Livesy (106).

2.5 Laboratory Measures

Baseline serum glucose and free fatty acids were assayed by established enzymatic procedures (Beckman Synchron CX7 or CX5; Beckman Coulter, Brea, CA) using the Wako FFA reagents (Richmond, VA). Baseline plasma insulin and C-peptide were measured on an Immulite autoanalyzer (DPC, Los Angeles, CA). Steady state (insulin infusion) serum glucose and insulin were assayed during the euglycemic-hyperinsulinemic clamp in the same manner as the baseline samples. The enzymatic assay was not sensitive enough to reliably measure insulin-suppressed free fatty acids (FFAs), so steady state (insulin infusion) FFAs were measured in triplicate by high performance liquid chromatography (HPLC) as previously described (107) by Dr. John Miles, Mayo Clinic, Rochester, MN.

2.6 Fat Cell Size

Fat cell size was determined as previously described (108). Briefly, adipose tissue was fixed in osmium tetrachloride/collidine-HCl followed by disassociation by urea digestion. Cells were sized and counted on a Multisizer-3 (Beckman Coulter, Fullerton, CA) using a 400-μm aperture (dynamic linear range, 12–320 μm) and reported as the mean of all adipocytes >22.5μm.

2.7 Animal Study

Male C57BL/6J mice were housed at room temperature with a 12h light-12h dark cycle for five weeks. Six mice ate the control diet 10% fat diet (Research Diets, Inc., D12450B, New Brunswick, NJ: 10% of energy from fat, 20% of energy from protein, 70% of energy from carbohydrate) and seven mice ate the 45% high fat diet (HFD) (Research Diets, Inc., D12451, New Brunswick, NJ: 45% of energy from fat, 20% of energy from protein, 35% of energy from carbohydrate). All animals ate the control diet *ad lib* for two weeks and then seven were

switched to the HFD for three additional weeks. The gastrocnemius muscles were dissected and snap-frozen in liquid nitrogen.

2.8 Preparation of RNA and DNA

Human and mouse total RNA from 50-100 mg vastus lateralis and gastrocnemius muscle, respectively, was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). Mouse muscle from 20-30 mg gastrocnemius was digested overnight in proteinase K (FisherBiotech, Houston, TX) at 55^{o}C. DNA was extracted the following day with phenol-chloroform. The quantity and the integrity of the RNA and DNA were confirmed by Agilent 2100 Bioanalyzer according to manufacturer's procedure (Agilent Technologies, Palo Alto, CA).

2.9 Preparation of Whole Cell Extracts

Murine muscle tissues were homogenized in buffer (50mM HEPES, pH 7.4, 2mM EDTA, 150mM NaCl, 30mM $NaPPO_4$, 10mM NaF, 1% Triton X-100, 10µL/mL protease inhibitor, 10µL/mL phosphatase I inhibitor, 10µL/mL phosphatase II inhibitor, and 1.5mg/mL benzamidine HCl). The whole homogenates were centrifuged for 25 minutes at 15,000*g*, and supernatants were stored at -80 °C prior to Western immunoblotting.

2.10 Oligonucleotide Microarrays

RNA sample pairs (2µg) from the ten subjects were labeled by reverse transcriptase with dCTP-Cy3 and dCTP-Cy5, respectively, and in the inverse order (dye swap) using MICROMAX TSA Labeling & Detection kit (Perkin-Elmer, Wellesley, MA). Equal amounts of labeled cDNA probes were hybridized in duplicate to oligonucleotide slides containing 18,861 spots corresponding to 17,260 unique oligonucleotides (Compugen, NJ) in hybridization chambers (GenomicSolutions, Ann Arbor, MI) for up to 72 hours at 42°C. Detection and washing were performed at room temperature according to manufacturer's protocol (Perkin-Elmer, Wellesley,

MA). Oligonucleotide chips were spotted on to poly-L-lysine slides using a GeneMachine OmniGrid microarrayer (GenomicSolutions, Ann Arbor, MI) equipped with a Stealth SPH32 printhead and Stealth SMP4 Micro Spotting Pins (Telechem Internation, Inc., Sunnyvale, CA). Oligonucleotides were stored in 384-well plates in 45% DMSO. Microarray slides were scanned using a GSI Lumonics ScanArray 5000 scanner (Perkin-Elmer, Wellesley, MA) at high intensities (~95% for Cy3, ~75% for Cy5) and low intensities (~55% for Cy3, ~35% for Cy5) applying ScanArray Express software and quantified using QuantArray (GenomicSolutions, Ann Arbor, MI). All subsequent microarray analyses were performed using SAS version 8.2 (SAS, Cary, NC). A robust local regression procedure (LOWESS) was performed to remove the systematic variations in the measured gene expression levels so that differences in expression across the samples could be distinguished accurately and precisely (109). After normalization, gene shaving (110) and bootstrapping (111), cluster analysis was performed (112), and the slide effect, dye effect and variety effect, as well as the duplicate design, were taken into account in an ANOVA model (113). Resampling-based multiple pairwise comparison was used to identify the differentially expressed genes before versus after the high fat diet. Differentially expressed genes were identified based on a Bonferroni

adjusted p-value < 0.001.

2.11 Real Time qRT-PCR for RNA

RNA sample pairs (1μg) were reverse transcribed using iScript cDNA synthesis kit (BioRad, Hercules, CA) and SYBR Green I qRT-PCR (Applied Biosystems, Roche, Branchburg, NJ). All primers and probes were designed using Primer Express version 2.1 (Applied Biosystems, Roche, Branchburg, NJ). The sequences of primers and probes and accession numbers for each gene are shown in Appendix A.1 and Appendix A.3. Real time RT-PCR

reactions (114) for tested genes were performed using the Taqman technique (Applied Biosystems, Roche, Branchburg, NJ). Real-time RT-PCRs were performed as one-step reactions in an ABI PRISM 7900 (Applied, Biosystems, Branchburg, NJ) using the following parameters: one cycle of 48°C for 30 min, then 95° C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. For all assays performed using SYBR Green I, 18S was used as the internal control, and for all assays performed using Taqman primers and probe, RPLP0, which is the human equivalent of the murine 36B4 (115), was used as the internal control. Cyclophilin B was used for all murine assays. All expression data were normalized by dividing the amount of target gene by the amount housekeeping gene used as an internal control.

2.12 Real Time qPCR for mtDNA and Genomic DNA Copy Number

Taqman primers and probes were designed using Primer Express version 2.1 (Applied Biosystems, Roche, Branchburg, NJ). The sequences of primers and probes and accession numbers for each gene are shown in Appendix A.1. Real-time PCR was carried out in an ABI PRISM 7900 sequence detector (Applied, Biosystems, Branchburg, NJ) using the following parameters: one cycle of 50°C for 2 min, then 95° C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Murine skeletal muscle genomic DNA copy number was measured at the UCP2 gene using a primer-probe set that amplifies genomic DNA. Mitochondrial DNA copy number was measured from the COXII mtDNA gene. Mitochondrial DNA copy number was calculated by first taking the absolute value of the delta Ct values between groups (control vs. HFD). Because amplification occurs exponentially (increasing two-fold with each cycle of PCR), log base 2 of delta Ct as the copy number for each sample (116).

2.13 Western Immunoblotting

Homogenates were run on a 10% PAGE, transferred to PDVF membranes, incubated with the primary antibodies PGC1α (#P3363, US Biological, Swampscott, MA) and Cytochrome C (#556433, BD Biosciences, San Jose, CA) and signal detected using the ECL detection system (Pierce, Rockford, IL). GAPDH (#4699-9555, Biogenesis, Kingston, NH) was used as an internal control and brown adipose tissue as positive control.

2.14 Skeletal Muscle Enzyme Activities

Skeletal muscle samples were diluted 20-fold and homogenized in extraction buffer (0.1 M KH_2PO_4/Na_2PHO_4, 2 mM EDTA, pH = 7.2). Citrate synthase (CS), Cytochrome C oxidase (COX) and beta-hydroxyacyl-CoA dehydrogenase (BHAD) activities were determined spectrophotometrically as previously described (117; 118). DNA was extracted from the same homogenate (total nucleic acid extraction, Epicentre, Madison, WI) and mtDNA measured to correct for differences in the content of mitochondria (119).

2.15 Statistical Analysis

Statistical analysis for all real time qRT-PCR and qPCR data in skeletal muscle was performed using two-tailed paired Student's t-test for before versus after HFD (human) and unpaired Student's t-test for low fat versus high fat diet (mouse) to establish main effects of the high fat diet intervention. Adipose tissue gene expression and clinical data were correlated using regressions. The change in respiratory quotient (ΔRQ) was divided into quartiles (quartile 1 = $\Delta RQ < 0.06$; quartile 2 = $0.06 < \Delta RQ \leq 0.08$; quartile 3 = $0.08 < \Delta RQ \leq 0.11$; quartile 4 = $\Delta RQ > 0.11$) to illustrate the range in metabolic flexibility within the cohort. ANOVA was used to test for differences in biopsy and blood parameters across quartiles of metabolic flexibility (ΔRQ), with post-hoc testing by mean equality contrast between different groups using the

Tukey-Kramer HSD; alpha = 0.05. Type I error rate was set a priori at $p < 0.05$.Analysis was performed using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA) and JMP version 5.0 (SAS, Cary, NC).

CHAPTER 3: HIGH-FAT DIET DOWNREGULATES OXPHOS GENES*

3.1 Introduction

Type 2 diabetes affects over 110 million people worldwide, and Type 2 diabetes associated with obesity is reaching epidemic proportions (120; 121). Many pathophysiological processes have been implicated in diabetes, such as pancreatic β cell dysfunction and defects in insulin signaling, carbohydrate utilization and mitochondrial metabolism (83; 122). At the molecular and structural level, mitochondrial biogenesis and mitochondrial function are altered in diabetes, as well as in insulin-resistant relatives of Type 2 diabetics (86; 123). At the ultra-structural level, a reduction in the number, location and morphology of mitochondria is strongly associated with insulin resistance (123). Two recent microarray studies have shown that genes involved in oxidative phosphorylation (OXPHOS) exhibit reduced expression levels in the skeletal muscle of Type 2 diabetics and prediabetics. These changes may be mediated by the peroxisome proliferator-activated receptor γ coactivator-1 (PGC1) pathway. PGC1α- and PGC1β-responsive OXPHOS genes show reduced expression in the muscle of patients with Type 2 diabetes (83; 84). In addition to the cellular energy sensor AMPK, the peroxisome proliferator-activated receptor (PPAR) cofactors PGC1α (124-126) and possibly PGC1ß (127) activate mitochondrial biogenesis and increase OXPHOS gene expression by increasing the transcription, translation and activation of the transcription factors necessary for mitochondrial DNA (mtDNA) replication. Similarly, PGC1α increases the transcription of enzymes necessary for substrate oxidation, electron transport and ATP synthesis. Morphological and functional

* published as *Diabetes* **54**: 1926-1933, 2005, with permission from Diabetes.

studies (85; 123; 128), combined with the recent microarray data, indicate that PGC1 is important in the development of Type 2 diabetes.

Rates of ATP synthesis, measured *in situ* with magnetic resonance spectroscopy, are decreased in subjects with a family history of diabetes before the onset of impaired glucose tolerance (85; 86). Based on these results, the prevailing view is that these defects have a genetic origin (86). One common feature of diverse insulin resistant states is an elevation in non-esterified fatty acids (129). This gave rise to the concept of 'lipotoxicity' and 'ectopic fat' (130) and shifted attention toward the adipose tissue and increased free fatty acid concentrations as a potential foundation for insulin resistance (129).

Excess dietary fat has been implicated in the development of obesity and diabetes (131). Even at energy balance high fat diets will increase the flux of fatty acids through skeletal muscle for oxidation. The purpose of these experiments was to identify the transcriptional responses in skeletal muscle to an *in vivo* isoenergetic high fat diet (HFD) in healthy young men using oligonucleotide microarrays. We found downregulation of genes encoding proteins in complex I and complex II of the electron transport chain after an acute isoenergetic high fat diet. Similarly, Cytochrome C (complex III protein) and Surfeit 1 (complex IV protein), PGC1α and PGC1β mRNA were also downregulated by the HFD. These changes were recapitulated and amplified in a murine model after a three-week HFD. These studies implicate increased fatty acid flux in the defects in OXPHOS genes observed in diabetes and the prediabetic/insulin-resistant state.

3.2 Results

The characteristics of the study population are presented in Table 3.1. Fasting glucose, insulin and free fatty acids were unchanged as the diet was switched from a

'standard' fat content (35%) to a high fat diet (50%) (Table 3.2). Intra-subject changes in skeletal muscle gene expression before vs. after three days eating a 50% high fat diet were identified using oligonucleotide microarrays and analyzed by sequential analysis that included LOWESS normalization, gene shaving, bootstrapping and ANOVA. A cluster analysis grouped

Table 3.1 Characteristics of the study population before the high-fat diet. Ten male subjects were chosen from the larger study population based on a high aerobic capacity and insulin sensitivity. BMI, body mass index; GDR, glucose disposal rate; FFA, free fatty acids; FCS, fat cell size.

Subject Characteristics	Mean ± SD
Age (y)	23.0 ± 3.1
Height (cm)	179.7 ± 6.3
Weight (kg)	78.9 ± 13.2
BMI (kg/m²)	24.3 ± 3.0
VO_2max (ml/kg/min)	48.8 ± 3.3
WHR (au)	0.88 ± 0.1
RQ (au)	0.87 ± 0.1
Fasting Glucose (ml/dl)	78.4 ± 4.7
Fasting Insulin (µl/ml)	4.6 ± 1.4
GDR (mg/kgFFM/min)	14.7 ± 4.1
FFA (mmol)	0.4 ± 0.1
Body Fat (%)	16.2 ± 3.2
FCS (µl)	0.93 ± 0.2

Table 3.2 Change in overnight fasting blood parameters after a 50% high fat diet at energy balance and fixed activity level. Values are presented as sample means ± SEM. BL = baseline, HFD = high fat diet (50%fat). Repeated measures ANOVA was used to test for time effects; P = 0.847, 0.130, 0.047 for glucose, insulin and FFA, respectively. When post-hoc comparisons of each HFD day vs. *average* of the two baseline values were significant ($p < 0.05$; Dunnetts adjustment) these values are marked with an asterisk.

Variable	BL1	BL2	HFD day 1	HFD day 2	HFD day 3
Glucose (mg/dl)	88.60 ± 7.38	89.77 ± 8.74	89.00 ± 9.76	89.40 ± 7.29	90.40 ± 9.03
Insulin (mIU/ml)	6.81 ± 3.02	6.98 ± 2.86	6.65 ± 3.00	6.07 ± 2.98 *	6.89 ± 2.67
FFAs (mmol)	0.26 ± 0.08	0.28 ± 0.10	0.22 ± 0.08	0.25 ± 0.08	0.27 ± 0.10

the regulated genes into five clusters based on three principal components (Figure 3.1). The three-day isoenergetic high fat diet significantly changed the expression of 298 genes

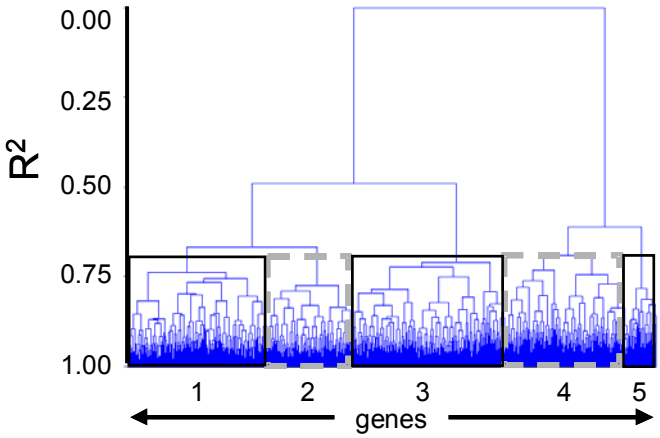


Figure 3.1 Cluster analysis of changes in gene expression with high-fat diet. In a cohort of healthy insulin-sensitive males (n=10), effect of a three-day isoenergetic high-fat diet (50% fat vs. 35% fat) reduced the expression of 298 genes in skeletal muscle. The 298 microarray 'hits' are divided among five clusters of co-regulated genes. Clustering was performed using the k-means technique. Differentially expressed genes were identified based on a Bonferroni adjusted *p*-value <0.001. The majority of the oxidative phosphorylation genes 'hits' were in cluster # 5.

($p < 0.001$; Appendix A.2). 163 genes were upregulated and 135 were downregulated by the high fat diet. Of those genes, six were known to be involved in oxidative phosphorylation by visual inspection or through gene ontogeny analysis ($p < 0.001$; Table 3.3). All of the oxidative phosphorylation genes were downregulated and none were upregulated. Three of these genes are components of complex I and one is a component of complex II. The remaining regulated gene is involved in mitochondrial solute transport.

We then sought to confirm the expression of these six oxidative phosphorylation genes by quantitative real time RT-PCR (Appendix A.1). Of the six 'hits' from the microarray all displayed the same downward trend with RT-PCR as by microarray, and three genes were 'confirmed' (Figure 3.2.A): NDUFB5 (3.19 ± 0.26 to 2.12 ± 0.20AU, $p < 0.01$), SDHB

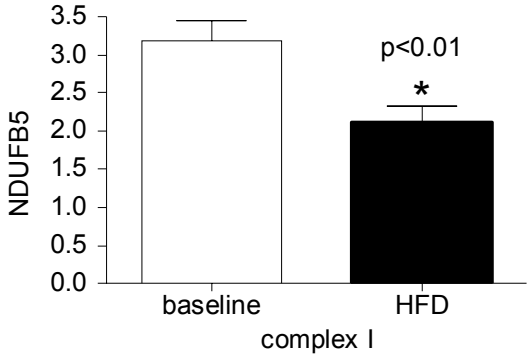
(0.26 ± 0.02 to 0.19 ± 0.02AU, $p < 0.05$), NDUFS1 (0.28 ± 0.03 to 0.21 ± 0.02AU, p=0.05), SLC25A12 (0.29 ± 0.04 to 0.19 ± 0.02AU, p=0.08), NDUFB3 (0.39 ± 0.05 to 0.26 ± 0.034AU, p=0.14) and NDUFV1 (0.36 ± 0.05 to 0.30 ± 0.04AU, p=0.32). The magnitudes of these

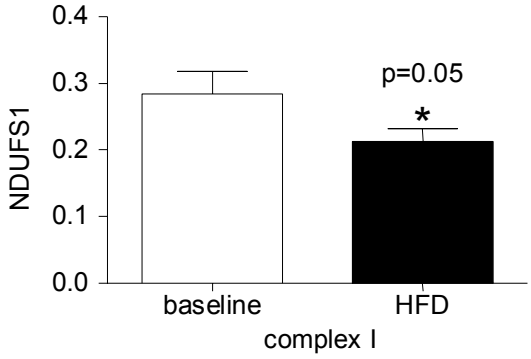
Table 3.3 Microarray hits - oxidative phosphorylation. By microarray analysis, 298 genes were up- or downregulated after a HFD. Of those 298, 6 are known to be involved in oxidative phosphorylation or mitochondrial function. NDUFB3, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3; NDUFB5, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5; SDHB, succinate dehydrogenase complex, subunit B; SLC25A12, solute carrier family 25 (mitochondrial carrier); NDUFV1, NADH dehydrogenase (ubiquinone) flavoprotein 1; NDUFS1, NADH dehydrogenase (ubiquinone) Fe-S protein 1.

Gene	Fold Change	p-value
NDUFB3	↓ 1.4	<0.01
NDUFB5	↓ 1.9	<0.01
SDHB	↓ 2.4	<0.01
SLC25A12	↓ 1.8	<0.01
NDUFV1	↓ 1.9	<0.01
NDUFS1	↓ 2.4	<0.01

changes (~ 20-30%) are strikingly similar to the decreases demonstrated by microarray analysis of reduced skeletal muscle oxidative phosphorylation gene expression found by Patti and Mootha in diabetic subjects (83; 84). As a subsequent step in elucidating the effects of the diet intervention on the expression of genes involved in mitochondrial function, we examined the mRNA for genes in complex III and complex IV using qRT-PCR (Figure 3.2.A). Cytochrome C (complex III) and Surfeit 1 (complex IV) expression levels were reduced (1.13 ± 0.07 to 0.85 ± 0.05AU, $p < 0.01$ and 1.10 ± 0.05 to 0.90 ± 0.05 AU, $p < 0.01$). Because expression levels of genes involved in mitochondrial function decreased, we examined gene expression of those involved in mitochondrial biogenesis. We observed a 20% and a 25% reduction in mRNA levels in PGC1α and PGC1β, respectively (Figure 3.3.A); PGC1α (1.44 ± 0.08 to 1.13 ± 0.06 AU,

Figure 3.2.A HFD decreases mRNA for genes involved in oxidative phosphorylation in healthy young men. Effect of a three day isoenergetic high fat diet (50% fat vs. 35% fat) in a cohort of healthy insulin-sensitive males (n=10) at baseline and after intervention on the expression of genes in complex I (NDUFB3, NDUFB5, NDUFV1, NDUFS1) complex II (SDHB), complex III (CYC1), complex IV (SURF1) and a mitochondrial carrier protein (SLC25A12). mRNA was quantified by qRT-PCR. Data are shown as means ± SE and corrected for the expression of 18S and RPLP0. NDUFB3 NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3; NDUFB5 NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5; NDUFV1 NADH dehydrogenase (ubiquinone) flavoprotein 1; NDUFS1 NADH dehydrogenase (ubiquinone) Fe-S protein 1); SDHB succinate dehydrogenase complex, subunit B; SLC25A12 solute carrier family 25 (mitochondrial carrier); CYC1 cytochrome c-1; SURF1 surfeit 1; 18S 18S ribosomal RNA; RPLP0 ribosomal protein, large, P0.


NDUFB5
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
p<0.01
*
baseline
HFD
complex I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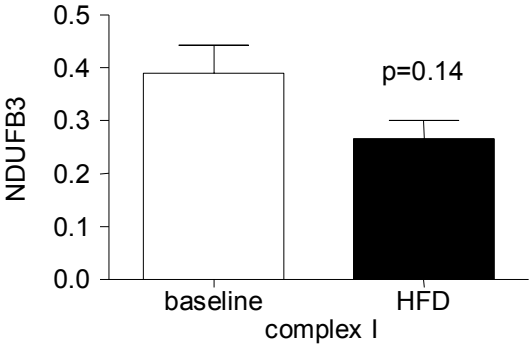
*
baseline
HFD
complex I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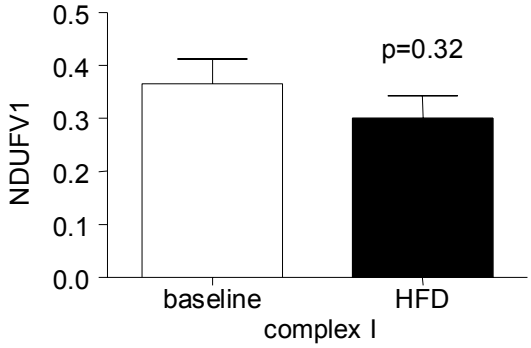
HFD
complex I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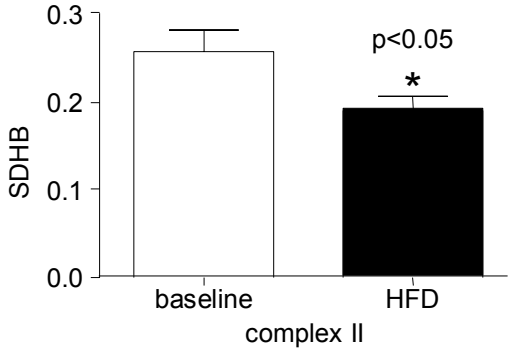
p=0.32
baseline
HFD
complex I


SDHB
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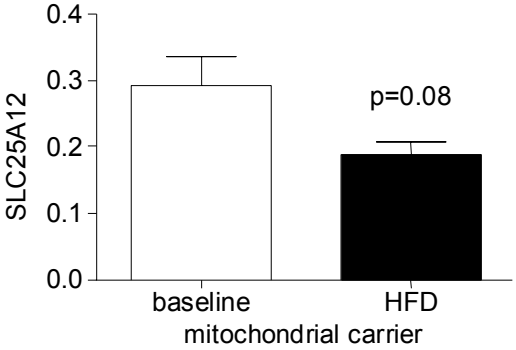
0.2
0.1
0.0
p<0.05
*
baseline
HFD
complex II


SLC25A12
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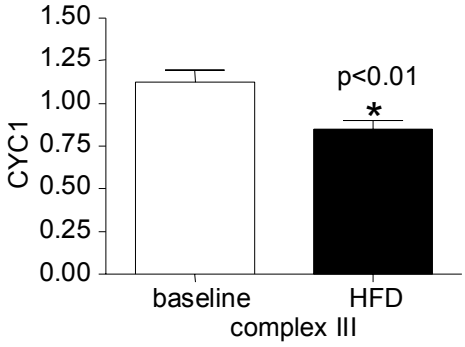
0.3
0.2
0.1
0.0
p=0.08
baseline
HFD
mitochondrial carrier


CYC1
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1.00
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0.50
0.25
0.00
p<0.01
*
baseline
HFD
complex III

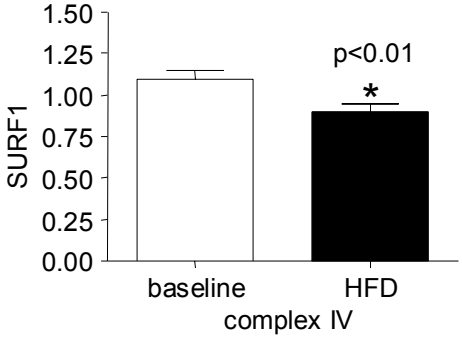
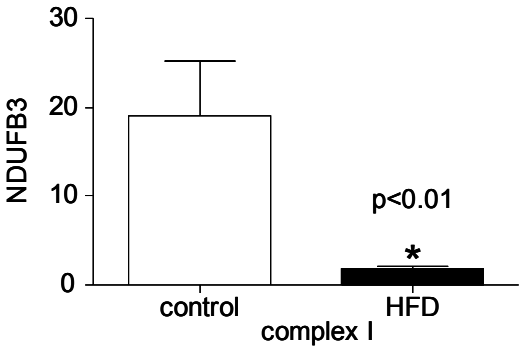
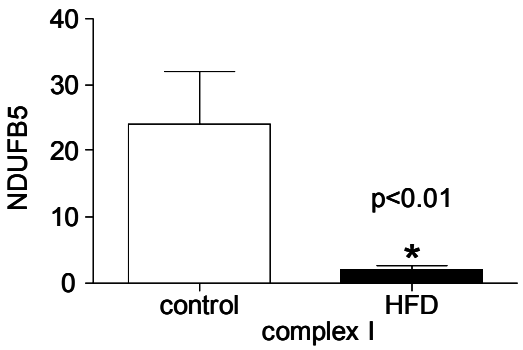

SURF1
1.50
1.25
1.00
0.75
0.50
0.25
0.00
p<0.01
*
baseline
HFD
complex IV

Figure 3.2.B HFD decreases mRNA for genes involved in oxidative phosphorylation in mice. Effect of a 21 day high fat diet (45% fat vs. 10% fat) in a cohort of C57Bl/6J mice (control, n=6; HFD, n=7) on genes in Complex I (NDUFB3 AND NDUFB5) Complex II (SDHB), Complex III (CYC1), Complex IV (SURF1) and a mitochondrial carrier protein (SLC25A12). Data are shown as means ± SE and corrected for the expression of Cyclophilin B. mm mus musculus; mmNDUFB3 NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3; mmNDUFB5 NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5; mmSLC25A12 solute carrier family 25 (mitochondrial carrier); mmSDHB succinate dehydrogenase complex, subunit B; mmCYC1 cytochrome c-1; mmSURF1 surfeit 1; mmCycB peptidylprolyl isomerase B (cyclophilin B).


NDUFB3
30
20
10
0
p<0.01
*
control
HFD
complex I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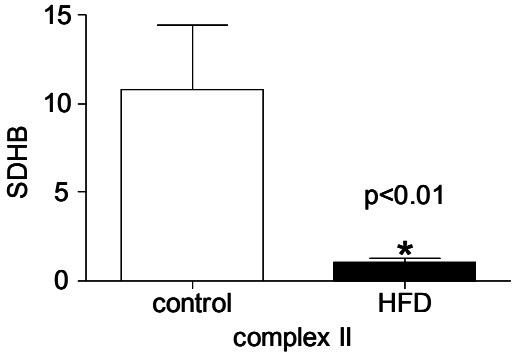
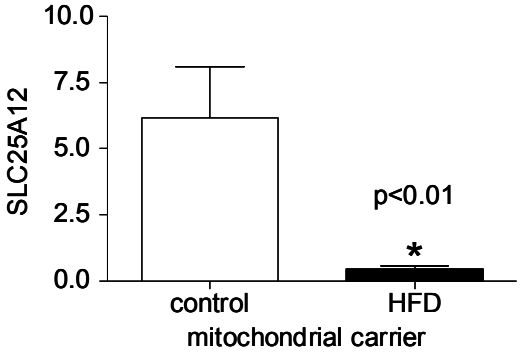
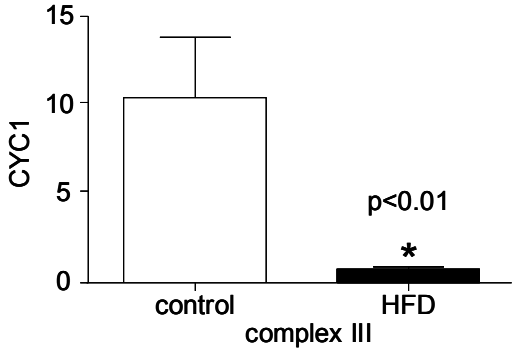
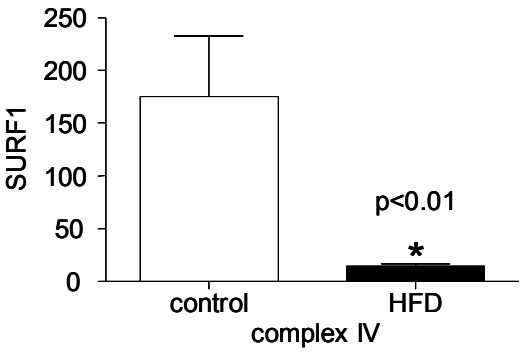
30
20
10
0
p<0.01
*
control
HFD
complex I


SDHB
15
10
5
0
p<0.01
*
control
HFD
complex II


SLC25A12
10.0
7.5
5.0
2.5
0.0
p<0.01
*
control
HFD
mitochondrial carrier


CYC1
15
10
5
0
p<0.01
*
control
HFD
complex III


SURF1
250
200
150
100
50
0
p<0.01
*
control
HFD
complex IV

p<0.01) and PGC1β (2.12 ± 0.16 to 1.59 ± 0.18 AU, p<0.05). Mitochondrial transcription factor A (TFAM), a key activator of mitochondrial transcription and its genome replication, was not significantly changed (2.00 ± 0.19 to 1.79 ± 0.19 AU, p=0.38), nor was nuclear respiratory factor 1, NRF1 (1.89 ± 0.13 to 1.56 ± 0.16AU, p=0.14) (Figure 3.3.A).

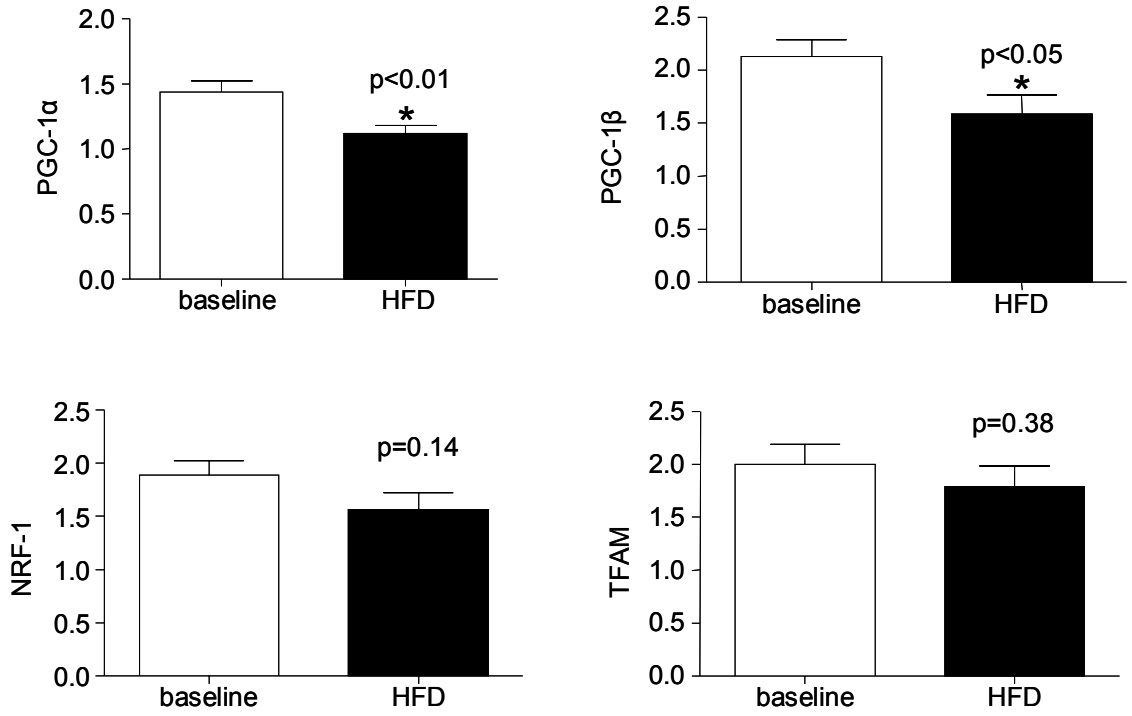


Figure 3.3.A HFD decreases expression of transcriptional cofactors involved in the regulation of oxidative phosphorylation gene expression and mitochondrial biogenesis in healthy young men. Effect of a three day isoenergetic high fat diet (50% fat vs. 35% fat) in a cohort of healthy insulin-sensitive males (n=10) at baseline and after intervention on the expression of genes involved in mitochondrial biogenesis. Data are shown as means ± SE and corrected for the expression of RPLP0. PGC1α peroxisome proliferative activated receptor, gamma, coactivator 1, alpha; PGC1β peroxisome proliferative activated receptor, gamma, coactivator 1, beta; TFAM transcription factor A, mitochondrial; NRF1 nuclear respiratory factor-1; RPLP0 ribosomal protein, large, P0.

We next tested whether the changes in gene expression we found in the clinic were present in a murine model of high fat diet-induced obesity. We fed C57Bl/6J mice either a 10% or 45% fat diet for three weeks. We chose two murine genes from complex I, one gene each from complexes II, III and IV and one mitochondrial carrier protein from the human

experiments. Decline in gene expression was of greater magnitude than that seen in the human experiments. As measured by real time qRT-PCR, each of these genes was downregulated in high fat fed mice as compared to their controls (Figure 3.2.B): NDUFB5 (24.05 ± 7.89 to 2.10 ± 0.44AU, $p < 0.01$), NDUFB3 (19.02 ± 6.25 to 1.82 ± 0.29AU, $p < 0.01$), SDHB (10.84 ± 3.58 to 1.05 ± 0.20AU, $p < 0.01$) SLC25A12 (6.14 ± 1.99 to 0.45 ± 0.11AU, $p < 0.01$), CYC1 (10.41 ± 3.40 to 0.79 ± 0.13 AU,$p<0.01$), and SURF1 (175.50 ± 57.35 to 13.81 ± 3.20 AU, $p<0.01$).

In parallel to the human experiment, we measured both PGC1α and PGC1β mRNA in these same mice. We found a 90% reduction in mRNA levels for both PGC1α and PGC1β (Figure 3.3.B): PGC1α (34.63 ± 12.57 to 2.67 ± 0.31 AU, $p<0.01$) and PGC1β (25.75 ± 9.03 to 1.85 ± 0.30 AU, $p<0.01$).

It has been shown that the half-life of a mitochondrion in a mammalian cell can range from about three days up to ten days, depending on the measurement technique (132). Given that the expression of both PGC1α and PGC1β were decreased, we hypothesized that skeletal muscle mitochondrial DNA copy number might be decreased by the high fat diet; however, we found no differences between animals fed a high fat diet when compared to those fed a low fat diet (Figure 3.3.B); mitochondrial DNA in low fat animals 1166 ± 112.50 and in high fat animals 1127 ± 70.28 AU, $p=0.78$.

3.3 Discussion

Numerous studies have implicated reduced mitochondrial biogenesis and oxidative phosphorylation in the pathogenesis of insulin resistance and Type 2 diabetes (133). Reductions in the expression of nuclear mRNAs encoding genes involved in mitochondrial oxidative phosphorylation have been reported in individuals with a family history of Type 2 diabetes and

in individuals affected by Type 2 diabetes (83; 84). These studies complement earlier anatomic and physiologic studies documenting alterations in mitochondrial number, mitochondrial enzyme

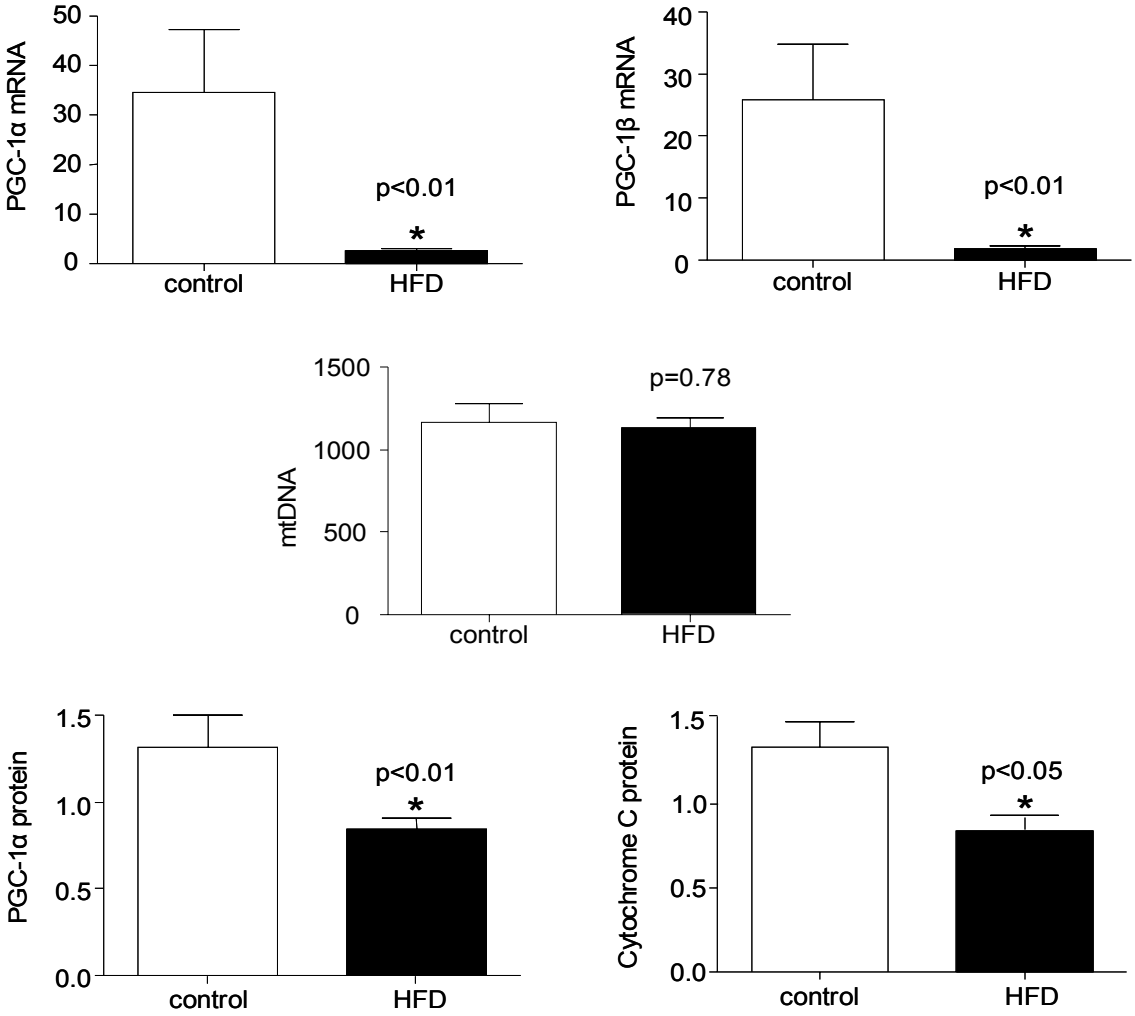


Figure 3.3.B HFD decreases expression of transcriptional cofactors involved in the regulation of oxidative phosphorylation gene expression and mitochondrial biogenesis in mice. Effect of a 21 day high fat diet (45% fat vs. 10% fat) in a cohort of C57Bl/6J mice (control, n=6; HFD, n=7) involved in mitochondrial biogenesis and mitochondrial DNA copy number per nuclear DNA copy number. Data are shown as means ± SE and corrected for the expression of Cyclophilin B. mmPGC1α peroxisome proliferative activated receptor, gamma, coactivator 1, alpha; mmPGC1β peroxisome proliferative activated receptor, gamma, coactivator 1, beta; mmCOXII cytochrome c oxidase II, mitochondrial; mmUCP2 uncoupling protein 2; mmCycB peptidylprolyl isomerase B (cyclophilin B).

activities and oxidative vs. glycolytic fiber type in skeletal muscle (123). Importantly, functional studies of skeletal muscle energy metabolism *in situ* demonstrate that ATP synthesis is reduced

in aging and the offspring of insulin resistant individuals (85; 86). The latter authors suggested that lipid flux is not involved in the reduction in expression of OXPHOS genes because fasting plasma NEFAs were similar in insulin-resistant and insulin-sensitive individuals, and glycerol turnover studies did not demonstrate an increase in lipolysis. However, our data suggest that dietary fat, or increased fat utilization in skeletal muscle, is an important factor in the observed reduction in OXPHOS genes in insulin resistant states. Microarray analysis and quantitative real time RT-PCR results revealed a downregulation of OXPHOS genes after eating the HFD, as well as transcription factors and cofactors, in our human experiments. Additionally, we have shown that the reductions in genes involved in oxidative phosphorylation and mitochondrial biogenesis were recapitulated in an animal model of dietary-induced obesity and insulin resistance (134) and were of a much greater magnitude in mice as compared to man.

Through the combined use of microarray technology, advanced bioinformatics and confirmation of the microarray results with qRT-PCR, we were able to identify subtle (20-30%) changes in OXPHOS gene expression without *a priori* grouping of genes based on known function (83). The advantage of this approach is genes that do not exhibit large changes in transcription, but that are clearly important in carbohydrate and energy metabolism (e.g. PGC1α), may be identified. Overall, an acute HFD elicits a coordinated downregulation of genes involved in oxidative phosphorylation within the electron transport chain. Moreover, the expression of PGC1β is also decreased.

Our results support the hypothesis that high fat diets and/or high fat flux through the mitochondria reduce the expression of nuclear genes encoding mitochondrial proteins and transcription factors involved in mitochondrial biogenesis. Both PGC1α and PGC1β were decreased by about 20% and were accompanied by a 20% reduction in OXPHOS gene

expression. Previous studies suggest a link between the downregulation of PGC1 and dysregulation of OXPHOS genes. Our results are consistent with this sequence of events, and indeed three of our OXPHOS genes found by microarray analysis were also present in the analyses of Mootha and Patti (83; 84). Therefore, our findings expand the view beyond the relationship between PGC1 and OXPHOS genes. We move upstream to show that increased fatty acid flux through the mitochondria decreases PGC1 expression and is associated with a downregulation of expression of genes involved in oxidative phosphorylation. Highly homologous to one another, PGC1α and PGC1β both alter mitochondrial metabolism but ultimately retain different physiological functions (135). Puigserver and Spiegelman (124) demonstrated that PGC1α is a master regulator of mitochondrial biogenesis and OXPHOS gene expression (136). PGC1α co-activation of NRF1-mediated transcription leads to transcription and subsequent translocation of TFAM to the mitochondrion, thus increasing mtDNA transcription (137; 138). In these studies, both coactivators PGC1α and PGC1β were downregulated; however, we saw no significant change in two downstream targets, NRF1 and TFAM (Figure 3.3.A).

Although an increase in free fatty acid concentrations was not seen in this cohort, fatty acid flux through the muscle is by necessity increased in these subjects as demonstrated by an increase in fat oxidation to match fat intake in this experimental paradigm (90). Another explanation for the reduction in the expression of these genes is that HFD decreases insulin-stimulated gene expression. Fatty acids decrease insulin signaling both *in vivo* and *in vitro*. Recent microarray studies demonstrate an upregulation of OXPHOS genes after a short-term insulin infusion (139). A reduction in insulin signaling might reduce expression of these same genes. Our studies do not identify the exact mechanism of the reduction in PGC1α, PGC1β or

their downstream targets. Rather these studies point toward dietary fat, or increased lipolysis, as a potential source of the previously reported reduction in mitochondrial oxidative phosphorylation and subsequent mitochondrial dysfunction.

Importantly, mice fed a HFD for three weeks showed a similar pattern of changes in gene expression as in the shorter (three day) human experiments. The magnitude of the changes in gene expression was much larger in mice than in man. In light of the fact that three days of HFD is not long enough to cause changes in mtDNA copy number as mitochondrial turnover is relatively slow (132), we next tested the hypothesis that the changes in the transcriptional cofactors (e.g. PGC1α and PGC1β) would decrease mitochondrial number in mice fed a HFD for three weeks. We found large changes in PGC1α and PGC1β, but mtDNA remained unchanged after three weeks of HFD in mice.

In some ways our inability to find changes in mitochondrial DNA copy number are inconsistent with recent studies demonstrating a reduction in mitochondrial number in diabetes and insulin resistance (116; 123) but similar to studies showing a decrease in OXPHOS gene expression (83; 84). One possibility is that 'chronic' versus 'acute' effects of high fat flux through mitochondria are different. In the prediabetic and diabetic states, increased lipid flux has been maintained for a longer period of time. Therefore, additional studies of mitochondrial number and function, including electron transport chain activity and electron microscopy, will be needed to fully rule out subtle changes in mitochondrial number or function with an acute HFD.

Our studies reveal a key question: "why would increased fatty acid flux decrease the expression of genes needed to oxidize these same fatty acids?". Fasting is another 'normal' physiological condition where fatty acid flux through skeletal muscle is increased. Surprisingly, fasting produces changes in gene expression that are strikingly similar to the pattern of fat-

induced changes observed in our studies of high fat diets. For example, Jagoe et al found that CASQ2 (calsequestrin 2), NDUFS1, glycogen synthase and PDK4 (pyruvate dehydrogenase kinase isoenzyme 4), four genes found on our microarray 'hit' list (Appendix A.2) and confirmed by qRT-PCR (data not shown), were similarly regulated by fasting in rodents (140). This may explain the paradoxical decrease in systems needed to oxidize fatty acids (nuclear genes encoding mitochondrial proteins, PGC1α) when fat flux is increased during a high fat diet. In other words, the parallel results between fasting and high fat diets suggest that fat flux through the skeletal muscle might be interpreted as a signal of fasting/starvation by the muscle cell itself. Signaling systems normally reserved for responding to energy deprivation (fasting) may be co-opted when dietary fat is increased. This hypothesis is also consistent with the observed changes in the transcription of genes involved in non-oxidative metabolism (e.g. glycolysis) found on our microarray 'hit' list (Appendix A.2).

In conclusion, high fat diets in both insulin-sensitive humans and in mice were associated with a reduction in the expression of genes involved in oxidative capacity (e.g. genes of the electron transport chain), nuclear genes encoding mitochondrial proteins (e.g. mitochondrial carrier proteins) and those involved in mitochondrial biogenesis (e.g. PGC1α and PGC1ß). These studies support the novel hypothesis that high fat diets or high fat flux explain the reduction in OXPHOS genes seen in aging, the prediabetic state and in overt diabetes.

CHAPTER 4: GLUCOSE METABOLISM VIA A LONG-TERM TRANSCRIPTIONAL LOOP*

4.1 Introduction

Type 2 diabetes affects over 110 million people worldwide, and Type 2 diabetes associated with obesity is reaching epidemic proportions (1, 2). Many pathophysiological processes have been implicated in diabetes, such as pancreatic β cell dysfunction, as well as defects in insulin signaling, carbohydrate utilization and mitochondrial metabolism (3, 4). The earliest detectable abnormality in people at risk for Type 2 diabetes is insulin resistance in skeletal muscle. The ability of insulin to activate signal transduction events, alter gene expression of selected genes (5) and stimulate muscle glycogen synthesis is an integral part of the body's response to macronutrient intake.

The physiologic purpose of alterations in fuel selection is to channel energy to and from the appropriate storage compartments under all circumstances that may confront the organism. An obligate need exists to regulate glycogen within a relatively narrow window. Studies conducted in subjects with Type 2 diabetes and in healthy subjects to determine the fate of glucose after it is taken up by muscle cells demonstrate that muscle glycogen synthesis, along with glycolysis, is an important pathway in skeletal muscle glucose metabolism (6). Muscle cells adjust the fuels they oxidize in order to match substrate supply, signals from the endocrine and neural systems and the ATP required for contraction.

* published as *Metabolism: Clinical and Experimental* **55**: 1457-1463, 2006, with permission from Metabolism.

The supply of substrate to muscle tissue depends upon several factors including the dietary macronutrient content, the storage of nutrients in liver and fat, capillary recruitment and transport, and in the case of triglycerides, the release of free fatty acids by the enzymatic activity of lipoprotein lipase (LPL) at the surface of the cell. The adjustment of substrate oxidation to the local nutrient concentrations occurs through at least three mechanisms: minute-to-minute changes in the activity of enzymes that direct carbohydrate and fat into oxidation or storage (6), the activation of signaling pathways such as PKC (8) and NFκB (9) and through long-term regulatory systems that involve changes in gene transcription and hence the cellular machinery driving the first two processes. The ability of insulin to regulate glucose homeostasis, i.e. insulin sensitivity, is closely related to these regulatory pathways, justifying further attempts to unravel the details of these regulatory systems. Strong experimental evidence exists for each of these pathways, particularly for the regulation of substrate utilization and insulin sensitivity through enzyme activity (7) and signaling pathways (8). Less data is available to implicate transcriptional pathways as key components of this autoregulatory loop. We previously described the effects of a short-term HFD to reduce the expression of genes related to oxidative phosphorylation within the skeletal muscle of young, healthy volunteers (10). The purpose of this research was to expand that investigation and to identify the transcriptional responses for other metabolic systems such as carbohydrate oxidation and storage, as well as fatty acid oxidation.

4.2 Results

Participants presented to the Pennington Biomedical Research Center inpatient unit (Figure 4.1) on day -4 at 07:00 PM. The characteristics of the study population are presented

in Table 4.1. Fasting glucose, insulin and free fatty acids were unchanged as the diet was switched from 'standard' fat : carbohydrate content (35% : 49%) to high fat : low

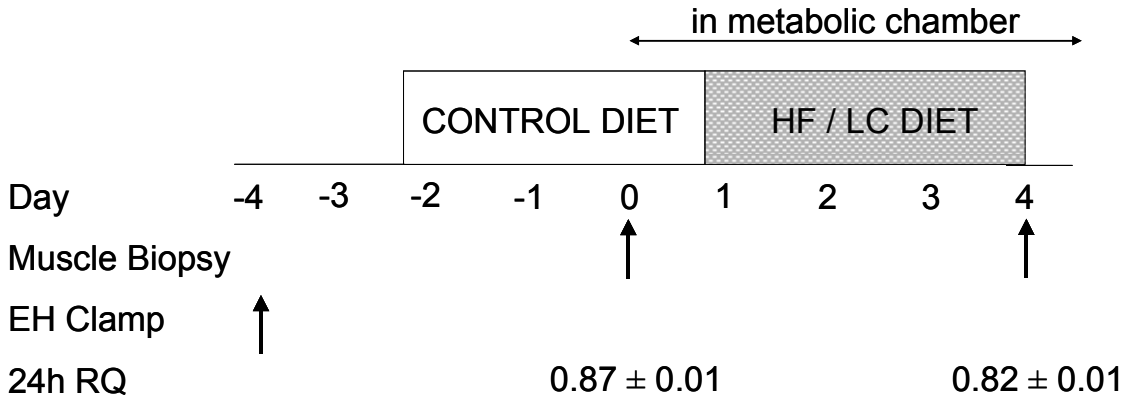


Figure 4.1 ADAPT study design. Subjects consumed a prescribed control diet (35% fat, 49% carbohydrate, 16% protein) followed by a euglycemic-hyperinsulinemic (EH) clamp and a muscle biopsy. They were switched to a prescribed HF/LC diet (50% fat, 34% carbohydrate, 16% protein) and placed in metabolic chamber for a total of 4 days where fatty acid oxidation was measured by respiratory quotient (RQ) at Day0 and Day4. They finished with a muscle biopsy on Day4.

carbohydrate content (50% : 34%; data not shown). Intra-subject changes in skeletal muscle gene expression before versus after three days eating the high fat/low carbohydrate diet were identified using oligonucleotide microarrays according to low laser analysis previously described (10).

The three-day isoenergetic high fat/low carbohydrate diet significantly changed the expression of 298 genes according to the low laser 'hit' list ($p < 0.01$; Appendix A.2). 163 genes were upregulated and 135 were downregulated by the high fat/low carbohydrate diet. A further analysis of the low laser dataset revealed an additional 72 genes regulated by the intervention ($p > 0.01 < 0.05$; Appendix A.4).

From these analyses, seven of those genes were known to be involved in glucose metabolism by visual inspection or through gene ontogeny analysis (Table 4.2). Of the

glucose metabolism genes five were downregulated and two were upregulated. Fructose-2,6-biphosphatase 3 (PFKFB3) and pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4) increased

Table 4.1 Characteristics of the study population before high fat/low carbohydrate diet. Ten male subjects were chosen from the larger study population based on a high aerobic capacity and insulin sensitivity. BMI, body mass index; GDR, glucose disposal rate; CHO, carbohydrate; FFA, free fatty acids.

Subject Characteristics	Mean ± SD	Range
Age (y)	23.0 ± 3.1	18.0 – 28.0
Height (cm)	179.7 ± 6.3	170.6 – 189.5
Weight (kg)	78.9 ± 13.2	61.7 – 104.8
BMI (kg/m^2)	24.3 ± 3.0	21.0 – 30.7
WHR (au)	0.88 ± 0.1	0.8 – 1.0
RQ (au)	0.87 ± 0.02	0.85 – 0.90
VO_2max (ml/kg/min)	49.4 ± 4.0	45.5 – 56.1
Fasting Glucose (mg/dl)	78.4 ± 4.7	70.0 – 86.0
Fasting Insulin (μU/ml)	4.6 ± 1.4	2.6 – 7.6
GDR (mg/kgFFM/min)	14.7 ± 4.1	9.9 – 24.5
Cumulative CHO Balance	139.9 ± 163.9	-208.8 – 297.5
FFA (mmol)	0.4 ± 0.1	0.07 – 0.50
Body Fat (%)	16.2 ± 3.2	11.5 – 20.4

at the RNA expression level, while glycogen synthase 1, muscle (GYS1), galactose-1-phosphate uridyltransferase (GALT), pyruvate dehydrogenase, beta subunit (PDHB), mitogen-activated protein kinase kinase 3 (MAP2K3) and UDP-glucose pyrophosphorylase 2 (UGP2) decreased in expression.

All of the genes are involved in the oxidation and storage of glucose, or glucose transporter expression (18). All qRT-PCR results are based on the seven genes from the initial low laser analysis ($p < 0.01$). Within this cohort of insulin-sensitive males, although circulating free fatty acid (FFA) concentrations did not increase (Figure 4.2.A), we were able to demonstrate

an increase in fatty acid oxidation on the HF / LCD as measured by a significant decrease in their respiratory quotient (RQ) during their four-day stay in the metabolic chamber (Figure 4.2.B): (0.87 ± 0.01 to 0.82 ± 0.01AU, $p < 0.01$). Due to phenotypic changes in the subjects as evidenced by a decrease in RQ, as well as displaying a positive carbohydrate balance subsequent to intervention, we then sought to confirm the expression of these glucose metabolism genes by real time qRT-PCR (Appendix A.3). Of the 'hits' from the low laser microarray list ($p < 0.01$), three genes were confirmed (Figure 4.3.A): PDK4 (0.09 ± 0.01 to 0.16 ± 0.03AU, $p < 0.01$), PFKFB3 (1.61 ± 0.26 to 3.28 ± 0.52AU, $p < 0.01$) and GYS1 (2.17 ± 0.17 to 1.71 ± 0.16AU, $p < 0.01$).

Table 4.2 Microarray hits in glucose metabolism pathways. By microarray analysis, a total of 370 genes were up- or downregulated after a HF/LCD. Of those 370, 7 are known to be involved in glucose metabolism. PFKFB3, fructose-2,6-biphosphatase 3; PDK4, pyruvate dehydrogenase kinase, isoenzyme 4; GYS1, glycogen synthase 1 (muscle); GALT, galactose-1-phosphate uridyltransferase; PDHB, pyruvate dehydrogenase, beta subunit; MAP2K3, mitogen-activated protein kinase kinase 3; UGP2, UDP-glucose pyrophosphorylase 2.

Gene	Fold Change	p-value
PFKFB3	↑ 3.3	< 0.01
PDK4	↑ 1.7	< 0.01
GYS1	↓ 1.7	< 0.01
GALT	↓ 1.6	< 0.01
PDHB	↓ 1.7	< 0.01
MAP2K3	↓ 2.0	< 0.01
UGP2	↓ 2.0	< 0.01

We next tested whether the changes in gene expression that we found in the clinic were present in a murine model of high fat/low carbohydrate diet-induced obesity. We fed

C57Bl/6J mice either a 10% : 70% or 45% : 35% high fat/low carbohydrate diet for three weeks. We chose two murine genes involved in glucose oxidation and two genes involved in glucose

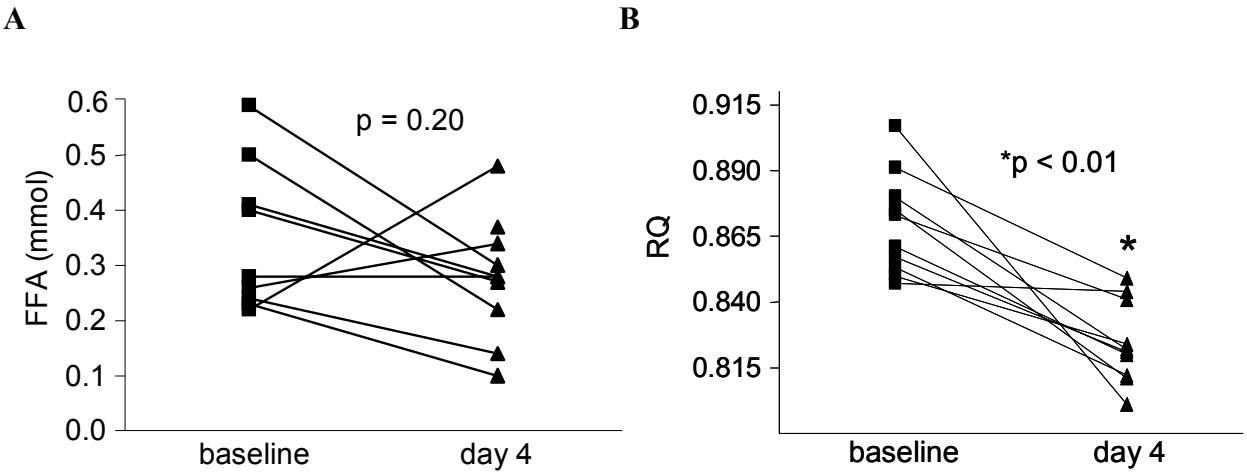


Figure 4.2 HF/LCD shifts substrate utilization toward fatty acid oxidation. (A) Effect of a three-day isoenergetic high fat/low carbohydrate diet (50% : 34% fat) in a cohort of healthy insulin-sensitive males (n=10) on circulating free fatty acid (FFA) concentrations during their stay in the metabolic chamber. (B) Effect of a three-day isoenergetic high fat/low carbohydrate diet (50% : 34% fat) in a cohort of healthy insulin-sensitive males (n=10) on fatty acid oxidation as measured by respiratory quotient (RQ) during their stay in the metabolic chamber.

storage from the human experiments. The changes in gene expression were in the same direction, but of a greater magnitude than those seen in the human experiments. As measured by real time qRT-PCR the results in rodents matched those in man (Figure 4.3.B): PDK4 (0.31 ± 0.03 to 1.05 ± 0.07AU, $p < 0.01$), PFKFB3 (0.65 ± 0.04 to 1.08 ± 0.17AU, $p < 0.01$), GYS1 (3.60 ± 0.66 to 0.66 ± 0.06AU, $p < 0.01$) and GALT (4.37 ± 0.89 to 0.81 ± 0.07AU, $p < 0.01$).

4.3 Discussion

During insulin stimulation the skeletal muscle is the major site for glucose disposal (19), thus defects in insulin-mediated glucose uptake and in its metabolic fate in this tissue are thought to be responsive to the macronutrient composition of the diet. Since Randle's proposal in 1963

(7) of a glucose-fatty acid cycle that embodies direct competition between substrates for mitochondrial oxidation, a plethora of mechanisms have been put forward to explain how fuel

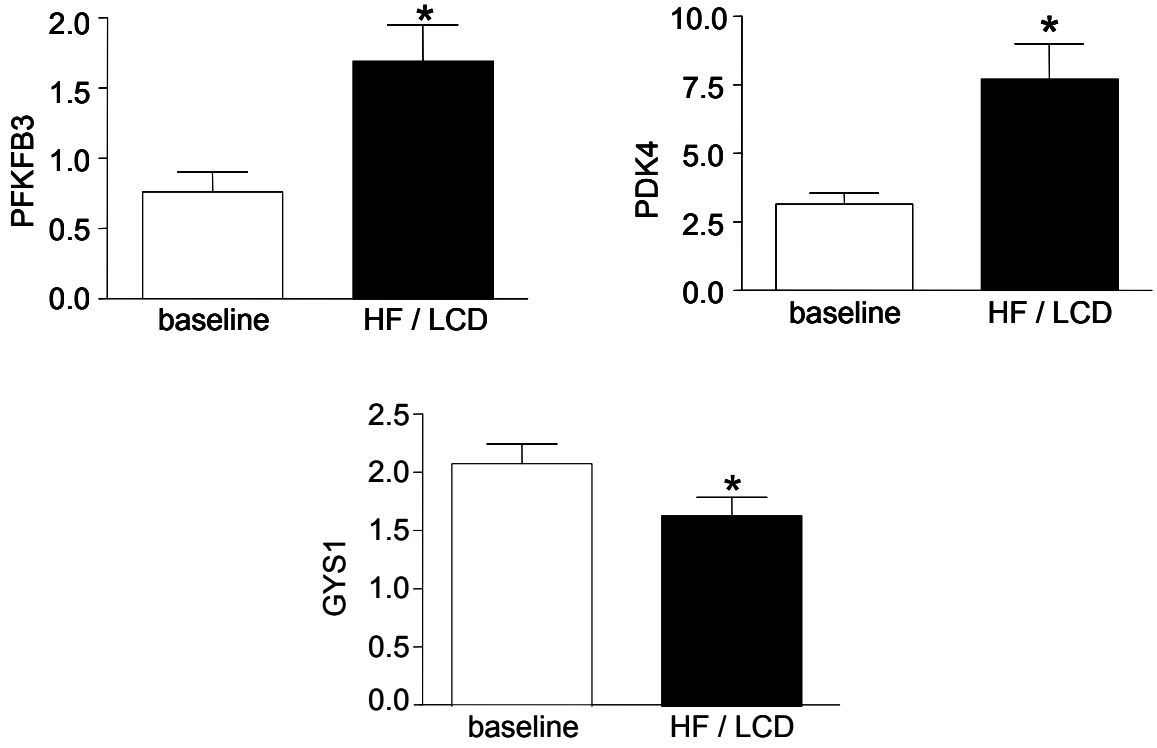


Figure 4.3.A HF/LCD regulates mRNA for genes involved in glucose metabolism in healthy young men. Effect of a three-day isoenergetic high fat/low carbohydrate diet (50% : 34% fat) in a cohort of healthy insulin-sensitive males (n=10) at baseline and after HF/LCD intervention on the expression of genes involved in glucose oxidation (PFKFB3 and PDK4) and glucose storage (GYS1). mRNA was quantified by qRT-PCR. Data are shown as means ± SE and corrected for the expression of RPLP0. PFKFB3 fructose-2,6-biphosphatase 3; PDK4 pyruvate dehydrogenase kinase, isoenzyme 4; GYS1 glycogen synthase 1 (muscle); RPLP0 ribosomal protein, large, P0.

Substrates could interfere with glucose disposal in skeletal muscle (20-22). *In vivo*, release and/or intermediate products of lipid fuels (fatty acids and ketone bodies, respectively) released into the circulation (e.g. in starvation or diabetes) may inhibit the catabolism of glucose in muscle, as well as its storage (8, 23).

Although an increase in free fatty acid concentrations was not seen in this cohort (Figure

4.2.A), fatty acid flux through the muscle is by necessity increased in these subjects as demonstrated by a decrease in 24h respiratory quotient (RQ; Figure 4.2.B) to match the fat

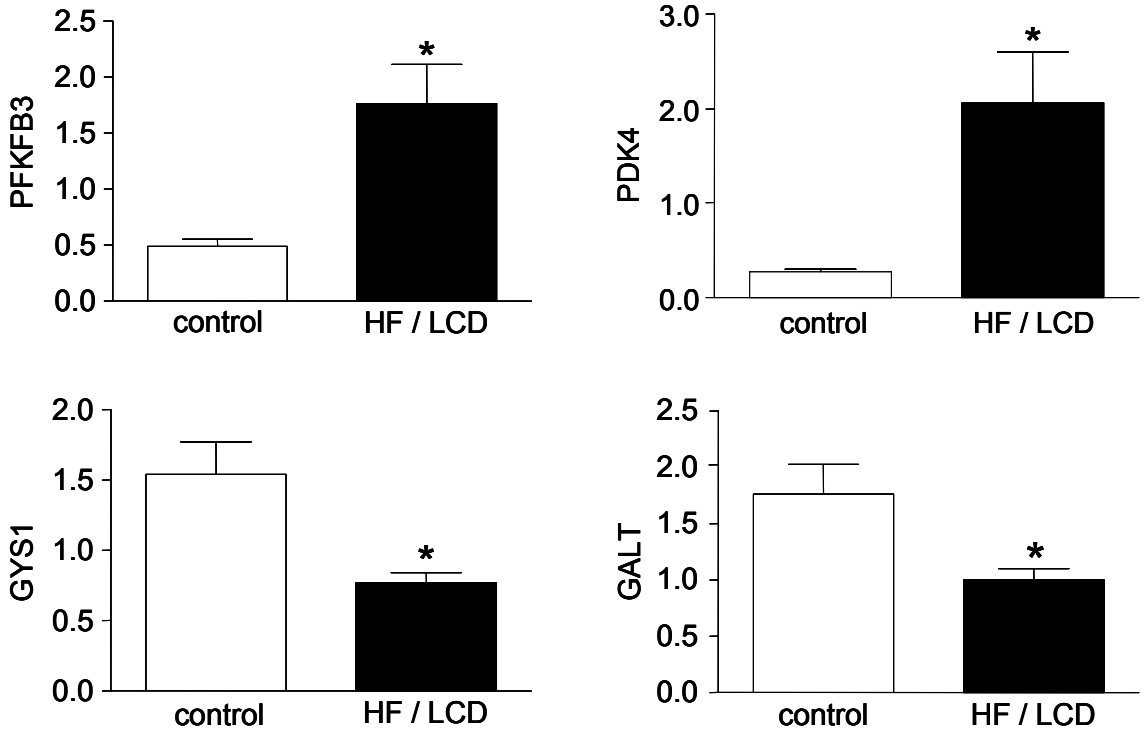


Figure 4.3.B HF/LCD regulates mRNA for genes involved in glucose metabolism in mice. Effect of a 21–day *ad libitum* feeding of a high fat/low carbohydrate diet (45% : 35%) in a cohort of C57Bl/6J mice (control, n=6; HF/LCD, n=7) on genes involved in glucose oxidation (PFKFB3 and PDK4) and glucose storage (GYS1 and GALT). Data are shown as means ± SE and corrected for the expression of Cyclophilin B. PFKFB3 fructose-2,6-biphosphatase 3; PDK4 pyruvate dehydrogenase kinase, isoenzyme 4; GYS1 glycogen synthase 1 (muscle); GALT galactose-1-phosphate uridyltransferase.

intake in this experimental paradigm (11). Therefore, substrate utilization is shifting more toward oxidation of fatty acids by reducing the oxidation of glucose; the uptake and utilization of glucose should consequently be reduced. A negative carbohydrate balance occurs immediately after consumption (one to two days) of a HF/LCD indicating a continued glucose oxidation and glycogen depletion as fat oxidation takes days to catch up to fat intake (31, 36). A positive

carbohydrate (CHO) balance was observed at the end of the three-day HF/LCD in this cohort of healthy young males, thus indicating a decrease in glucose oxidation (Table 4.1). We also

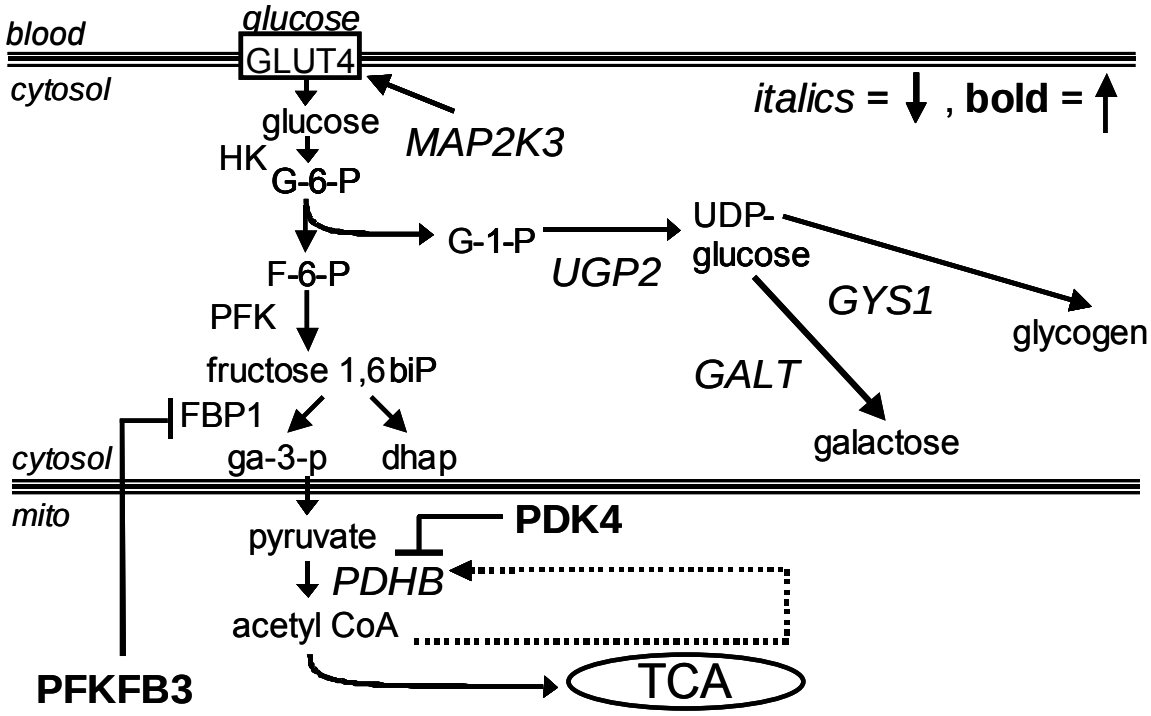


Figure 4.4 Transcriptional regulation of glucose flux. Upon entry into the muscle cell, glucose can either be oxidized via glycolysis and enter the TCA cycle or it can be stored as glycogen and galactose via non-oxidative glucose metabolism. The 9 genes that were altered by a three-day HF/LCD in human skeletal muscle according to low laser microarray analysis are marked blue if downregulated and red if upregulated. PFKFB3 fructose-2,6-biphosphatase 3; PDK4 pyruvate dehydrogenase kinase, isoenzyme 4; GYS1 glycogen synthase 1 (muscle); GALT galactose-1-phosphate uridyltransferase; PDHB pyruvate dehydrogenase, beta subunit; UGP2 UDP-glucose pyrophosphorylase 3; MAP2K3 mitogen-activated protein kinase kinase 3; FBP1 fructose-1,6-biphosphatase; HK hexokinase; PFK phosphofructokinase.

observed a decrease in the messenger RNA levels of a number of genes involved in the oxidative, as well as the non-oxidative, glucose metabolism. Even more striking is the matching murine data showing a greater magnitude of change with a longer exposure to a high fat/low carbohydrate diet (three days versus three weeks). The extent of the effects of high fat/low carbohydrate dietary intake on muscle glucose metabolism reflects the length of each model's

exposure to the diet. For example, the human cohort responded with about a 30% change in gene expression after three days of high fat/low carbohydrate diet; whereas, the murine model had about a 70% change in gene expression with three weeks of exposure. These results indicate that the effects of the HF/LCD are not transient. Even more compelling is the concurrent change in phenotype, such as decreased RQ (Figure 4.2.B), positive CHO balance (Table 4.1) and the changes in the genes controlling glucose metabolism in response to the perturbation of this diet (Table 4.2).

A variety of model systems have largely confirmed the validity of Randle's experiments depicting the mechanism of substrate competition through changes in enzymatic activity as a result of product feedback inhibition, at least in terms of the acute effects of fatty acids on muscle glucose metabolism and storage (24, 25). Our results supplement those of Randle by demonstrating a downregulation of genes involved in glucose oxidation and storage when fat intake is increased and carbohydrate intake is decreased. Our findings relate to changes in mRNA expression only, and we acknowledge that many of the enzymes involved in glycolysis and glycogen synthesis are regulated allosterically, and through covalent modification. Transcriptional regulation (Figure 4.4) may enhance the Randle cycle or changes in the activation of signaling molecules / pathways such as PKC or ceramides (8, 21).

Through the use of bioinformatics and microarrays, we expand the view of the glucose-fatty acid cycle beyond enzymatic activity to the level of gene transcription. In our analyses of the low laser microarray data set (Appendix A.2 and Appendix A.4), we found over 300 genes regulated by a three-day high fat/low carbohydrate diet in our human subjects. Four of our glucose metabolism genes (PFKFB3, PDK4, PDHB and GALT) were found in the analyses, and two of the four genes were confirmed by qRT-PCR. This is consistent with the

noise inherent to current microarray technologies (26, 27). After reporting changes in expression of the genes in the glucose metabolism gene cluster, we are left with the question of how the macronutrient composition of the diet affects other genes. The HF/LCD significantly regulated the expression of many OXPHOS genes, a few genes involved in fatty acid metabolism and various other genes known and unknown (Appendix A.2 and Appendix A.4).

Recent studies suggest that both acute and chronic increases in fatty acids downregulate oxidative phosphorylation in skeletal muscle (10, 23). Cluster analysis suggests that the changes in OXPHOS genes and the carbohydrate metabolism genes described herein are involved in a long-term transcriptional loop; however, the identity of the overall transcriptional control system remains unknown. It has been suggested that PGC1α plays a pivotal role as a link between a high fat diet and oxidative phosphorylation (87). Furthermore, the implication that both OXPHOS and carbohydrate metabolism genes are regulated at the transcriptional level is important for two reasons: (1) the unidentified transcriptional control system may be a 'master regulator' of energy homeostasis for both glucose and mitochondria and (2) strategies to identify the key regulators of this system should include both subsets of these genes.

Consonant with recent evidence by Pehleman et al (28) demonstrating the enzymatic regulation of glucose disposal in human skeletal muscle after a high fat/low carbohydrate diet, our results expand this view to a transcriptional co-regulation of glucose metabolism in this same tissue, as well as murine skeletal muscle after a high fat/low carbohydrate diet. Pehleman et al found a dramatic decrease in the activity of pyruvate dehydrogenase (PDH) with a concomitant increase in the enzymatic activity of PDH kinase (PDK) in response to a 56h eucaloric high fat/low carbohydrate diet (73% : 5%) within a healthy human cohort of insulin-sensitive males. While our results are not as dramatic, probably due to difference in dietary fat/carbohydrate

content (50% : 34% vs. 73% : 5%), we do show the same effects on PDK at the level of transcription. Together, our results identify both enzymatic activity and gene expression as targets of the transcriptional regulation.

Several studies over the past decade have shown pyruvate dehydrogenase kinase 4 (PDK4) message, protein and activity decrease as a result of increased free fatty acids, starvation and diabetes (30-32). More recently, PDK4 has become the target of diabetic drug investigations (33, 34). PDK4 promotes gluconeogenesis and suppresses glucose oxidation and maintains glucose levels in starvation. Although prior studies bolster the credibility of PDK4 as a diabetic drug target, our result argues against this view. We demonstrate that while glucose oxidation is suppressed, the transcription of genes before and after PDK4 is downregulated by HFD. Thus, the inactivation of PDK4 might not be effective in the setting of HFD / 'lipotoxicity'.

The original studies which formed the basis of the glucose-fatty acid cycle (7, 22, 35, 36) elucidated (1) fatty acid-induced desensitization of insulin-mediated glucose transport and (2) inhibitory effects of increased fatty acid oxidation on glucose metabolism. According to the Randle hypothesis (7, 24), an increase in lipid oxidation in muscle will decrease glucose oxidation by suppression of the mitochondrial pyruvate dehydrogenase complex, with a consequential reduction of glycolytic flux resulting in an increase in glucose-6-phosphate, inhibition of hexokinase activity and ultimately leading to decreased glucose uptake. Non-oxidative metabolism (storage) of glucose is, therefore, also predicted to be decreased. This mechanism has been reexamined and evolved over the past few decades (37), but the cellular effect is consistently observed. Differences in glucose-6-phosphate levels and mechanisms aside, a consistently observed effect of increased fat intake is the reduction of non-oxidative glucose metabolism (38). This data provides a basis for a transcriptional switch to regulate

substrate utilization within the skeletal muscle in response to changes in dietary macronutrient content. Overall, the novel contribution of this paper is its identification of multiple regulated genes within the same pathway by high fat/low carbohydrate diet.

CHAPTER 5: A ROLE FOR ADIPOSE TISSUE INFLAMMATION IN METABOLIC INFLEXIBILITY

5.1 Introduction

A key feature of the Type 2 diabetes (T2D) is metabolic inflexibility, which is characterized by impaired fasting fat oxidation and decreased insulin-stimulated glucose oxidation. Metabolic inflexibility is defined as an inability to transition between the utilization of lipids (fasting) and carbohydrates (after a meal). In obese individuals key features of metabolic inflexibility are (1) failure of skeletal muscle to appropriately move between use of lipid in the fasting state and use of carbohydrate in the insulin-stimulated state and (2) impaired transitioning from fatty acid efflux to storage (71). Obesity, hyperglycemia, impaired insulin stimulation of skeletal muscle glucose uptake (141), impaired mitochondrial biogenesis and decreased capacity for oxidation of dietary fat are all involved in the state of metabolic inflexibility (72). Furthermore, these physiologic characteristics are enriched in healthy young men with a family history of Type 2 diabetes (Ukropcova, B; in review).

White adipose tissue (WAT) is a major site of energy storage and is important for energy homeostasis: it stores energy in the form of triglycerides during nutritional abundance and releases energy as FFAs during nutritional deprivation (142; 143). While WAT provides a survival advantage in times of starvation, excess WAT and adipocyte hypertrophy are now linked to obesity-related health problems in the current nutritionally rich environments of developed countries. Furthermore, while adipocytes are traditionally known as a fat storage depot, they have recently been recognized as an endocrine organ that secretes hormones and cytokines as a metabolic response. Increased basal/fasted lipolysis in white adipose tissue leads to increased fasting FFA levels, which have been linked to insulin resistance (57). Increased chemotaxis and macrophage content in WAT are characteristics of the obese state. These

disturbances make the dysfunctional adipocyte a potential upstream 'causal' factor in metabolic inflexibility.

Taken together, this suggests that the quantity (body fatness) and the characteristics of the adipose tissue (adipocyte hypertrophy, disordered lipid metabolism, inflammation) might contribute to metabolic inflexibility. To explore this hypothesis, we studied 56 healthy young men under carefully controlled conditions, examining how adipose tissue mass and quality influence metabolic flexibility during a euglycemic-hyperinsulinemic clamp.

5.2 Results

Subject characteristics are listed in Table 5.1. All subjects were healthy young males ranging widely in BMI and percent body fat (20.1 – 34.7 kg/m^2 and 8.4 – 32.3 %, respectively). The respiratory quotient was measured before and during the euglycemic-hyperinsulinemic clamp (EHC) to determine metabolic flexibility (ΔRQ). ΔRQ varied greatly ranging from inflexible to flexible (0.03 – 0.25). Lower ΔRQ was associated with higher percent body fat (ANOVA, $p < 0.05$; Figure 5.1.A) and larger fat cell size (ANOVA, $p = 0.05$; Figure 5.1.B). Lower serum adiponectin levels were associated with lower ΔRQ (ANOVA, $p < 0.05$; Figure 5.1.C).

Obesity and hypertrophic adipocytes are associated with increased fasting FFAs and insulin-resistant adipocytes. To determine the contribution of disturbances in lipid metabolism, we explored the relationship between fasting and insulin-suppressed FFAs and metabolic flexibility. ΔRQ was not related to fasting FFAs (ANOVA, $p > 0.05$; Figure 5.1.D); however, higher concentrations of insulin-suppressed FFAs after a 2h insulin infusion were associated with a lower ΔRQ (ANOVA, $p < 0.05$; Figure 5.1.E). Fat cell size (FCS) was positively correlated

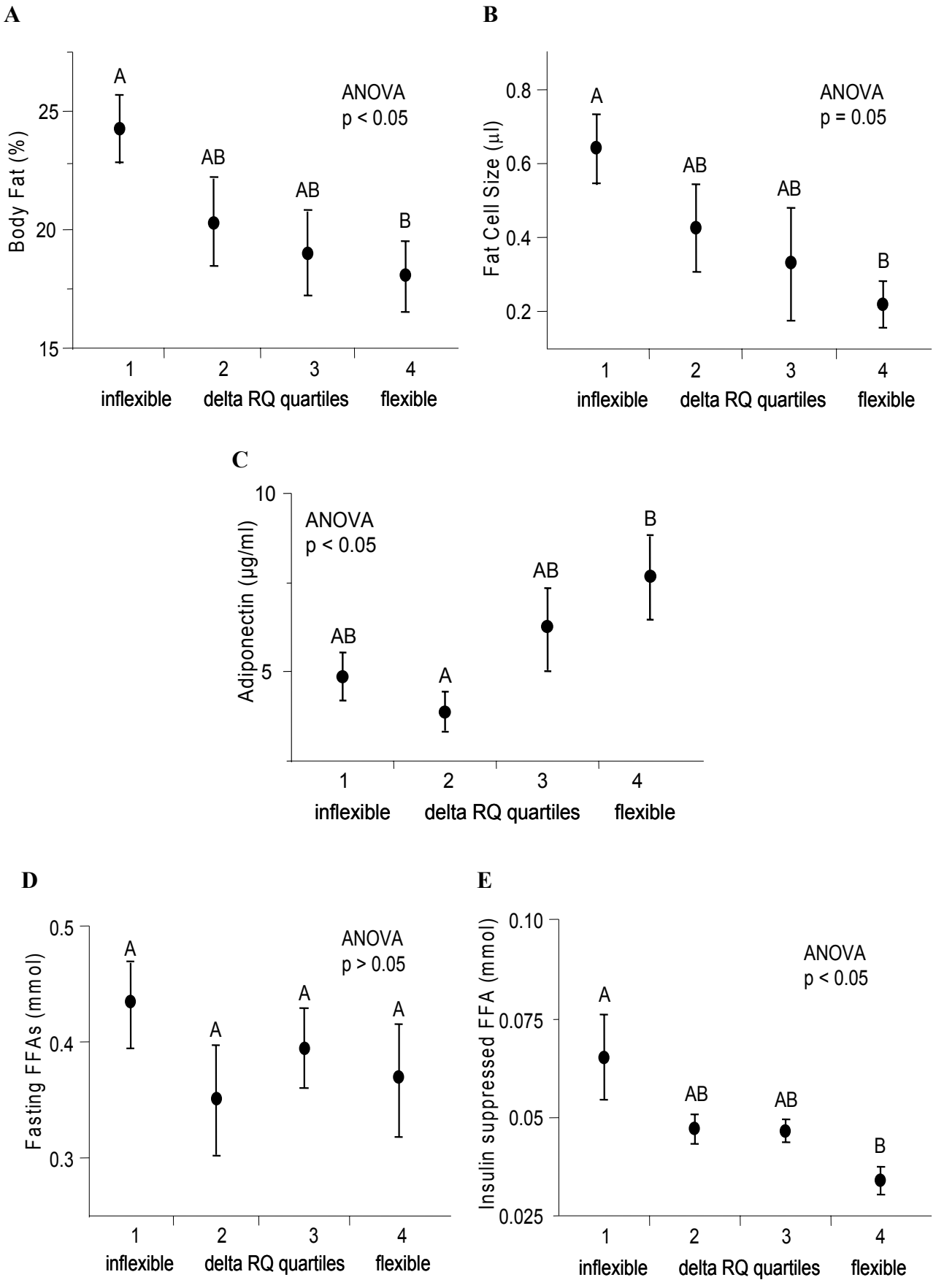
with fasting and insulin-suppressed free fatty acids (FFAs) during euglycemic-hyperinsulinemic clamp (EHC) (data not shown).

Table 5.1 Characteristics of the study population. All male subjects were chosen from the larger mixed gender study population. *during insulin infusion (80mIU/m^2BSA/min). BMI, body mass index; GDR, glucose disposal rate; WHR, waist to hip ratio; FFA, free fatty acids.

Subject Characteristics	Mean ± SD	Range
Age (y)	22.6 ± 3.2	18.0 – 29.0
Height (cm)	176.9 ± 5.8	163.0 – 189.5
Weight (kg)	82.5 ± 13.2	59.2 – 118.3
BMI (kg/m^2)	26.4 ± 4.1	20.1 – 34.7
WHR (au)	0.87 ± 0.07	0.7 – 1.0
Body Fat (%)	20.3 ± 6.5	8.4 – 32.3
Visceral adipose tissue mass (kg)	2.1 ± 1.3	0.5 – 5.7
Fasting RQ (au)	0.84 ± 0.04	0.74 – 0.95
Steady State* RQ (au)	0.93 ± 0.04	0.85 – 1.03
ΔRQ (au)	0.07 ± 0.15	-0.96 – 0.25
VO_2max (ml/kg/min)	41.2 ± 7.2	23.5 – 59.2
Fasting Glucose (mg/dl)	80.5 ± 5.4	66.0 – 90.0
Steady State* Glucose (mg/dl)	89.02 ± 4.5	78.0 – 102.0
Fasting Insulin (μU/ml)	8.2 ± 4.6	2.6 – 22.4
Steady State* Insulin (μU/ml)	160.1 ± 35.7	103.9 – 251.3
C-peptide (ng/ml)	1.93 ± 0.70	0.9 – 3.5
GDR (mg/kgFFM/min)	11.1 ± 4.1	4.0 – 24.5
Fasting FFA (mmol)	0.6 ± 0.2	0.07 – 0.84
Steady State* FFA (mmol)	0.05 ± 0.02	0.02 – 0.16
Fat cell size (μl)	0.6 ± 0.2	0.22 – 0.95

Given the relationship between adipocyte hypertrophy, disordered lipid metabolism and metabolic inflexibility, we examined mRNA expression of candidate genes involved in lipid metabolism (lipid synthesis, uptake, oxidation and storage, as well as lipolysis) and inflammation (chemokines and macrophage markers) and related these to insulin-suppressed free fatty acids (FFAs) and metabolic flexibility (change in respiratory quotient; ΔRQ) (Table 5.2). Insulin-suppressed FFAs were significantly correlated with the expression of genes involved in

Figure 5.1 Body fatness, FFAs, and adiponectin are related to metabolic inflexibility (ΔRQ) in healthy young men. Change in respiratory quotient (ΔRQ; metabolic flexibility) was measured before and during a euglycemic-hyperinsulinemic clamp (EHC) in the population of 56 healthy young men. ΔRQ was subdivided into quartiles and correlated with clinical measurements. Lower ΔRQ was associated with higher percent body fat (A), and larger fat cell size (B). Lower serum adiponectin levels were associated with lower ΔRQ (C). Lower ΔRQ was not related to fasting FFAs as measured by enzyme assay (D); however, lower ΔRQ was associated with higher levels of insulin-suppressed FFAs during steady state of the EHC (E) as measured by high performance liquid chromatography (HPLC). ANOVA was used to test for differences in biopsy and blood parameters across quartiles of metabolic flexibility (ΔRQ), with post-hoc testing by mean equality contrast between different groups using the Tukey-Kramer HSD; alpha = 0.05. Type I error rate was set a priori at $p < 0.05$. Data are shown as means $\pm$ SE. All Levels not connected by same letter are significantly different.


A
Body Fat (%)
25
20
15
A
AB
AB
B
ANOVA
p < 0.05
1
2
3
4
inflexible
delta RQ quartiles
flexible
B
Fat Cell Size (µl)
0.8
0.6
0.4
0.2
A
AB
AB
B
ANOVA
p = 0.05
1
2
3
4
inflexible
delta RQ quartiles
flexible
C
Adiponectin (µg/ml)
10
5
AB
A
AB
B
ANOVA
p < 0.05
1
2
3
4
inflexible
delta RQ quartiles
flexible
D
Fasting FFAs (mmol)
0.5
0.4
0.3
A
A
A
A
ANOVA
p > 0.05
1
2
3
4
inflexible
delta RQ quartiles
flexible
E
Insulin suppressed FFA (mmol)
0.10
0.075
0.05
0.025
A
AB
AB
B
ANOVA
p < 0.05
1
2
3
4
inflexible
delta RQ quartiles
flexible

chemotaxis (MCP-1 and MIP-1α) and markers of macrophage content (CD68 and MAC-2) (Table 5.2).

Metabolic flexibility (ΔRQ) was negatively correlated with MCP-1, MIP-1α, CD68 and MAC-2 (Table 5.2). The expressions of these four genes were highly

Table 5.2 Relationships between adipose tissue gene expression, glucose disposal, fasting free fatty acids and insulin-suppressed free fatty acids (R^2). All data are presented as R^2. *p < 0.01, **p ≤ 0.05. § during insulin infusion (80mIU/m^2BSA/min). GDR, glucose disposal rate; FCS, fat cells size; FFA, free fatty acids; ΔRQ, delta respiratory quotient; LPL, lipoprotein lipase; FAS, fatty acid synthase; PCK1, phosphoenolpyruvate carboxykinase 1; SCD1, stearoyl-CoA desaturase; MCAD, acyl-Coenzyme A dehydrogenase C-4 to C-12 straight chain; CAP, cbl-associated protein; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein 1, alpha subunit.

Functional Category	Gene Expression (mRNA)	GDR	% Fat	FCS	Fasting FFAs	Steady State FFAs§	ΔRQ
Lipid Uptake	CD36	0.07	-0.02	-0.01	-0.06	0.00	0.00
	LPL	0.00	0.00	0.08	-0.03	0.00	-0.06
Lipid Synthesis	FAS	0.07	-0.06	-0.05	0.00	**-0.13****	0.05
	PCK1	0.08	-0.07	-0.06	-0.08	0.00	0.00
	PPARγ1	**0.19***	-0.05	-0.05	**-0.16***	-0.01	0.00
	PPARγ2	-0.02	0.06	0.05	0.04	0.00	0.01
Lipid Storage	SCD1	0.06	-0.05	-0.01	0.00	-0.06	0.06
Lipid Oxidation	MCAD	**0.17***	**-0.08****	-0.06	**-0.14****	-0.01	0.01
	PPARa	**0.14****	**-0.10****	-0.07	**-0.10****	-0.01	0.00
Lipolysis	perilipin	0.07	-0.01	0.00	-0.02	-0.01	0.00
	CAP	**0.10****	-0.02	0.00	-0.03	-0.03	0.01
	ATGL	**0.11****	-0.02	-0.02	-0.01	-0.01	0.00
	HSL	**0.19***	**-0.08****	-0.05	**-0.08****	-0.03	0.00
Inflammation	CD68	-0.02	0.07	**0.10****	0.04	**0.38***	**-0.10****
	MAC-2	-0.02	0.08	0.07	0.01	**0.20***	**-0.10****
	MCP-1	0.00	0.04	0.04	0.01	**0.23***	**-0.11****
	MIP-1a	**-0.10****	**0.20***	**0.18***	**0.10****	**0.20***	**-0.16***

intercorrelated with R^2 ranging from 0.35 – 0.78 (data not shown). FCS was positively correlated with the chemokine MIP-1α (data not shown) and the macrophage marker

CD68 (data not shown). There was no relationship between fasting FFAs and the chemokines MCP-1 and MIP-1α; however, insulin-suppressed FFAs were positively

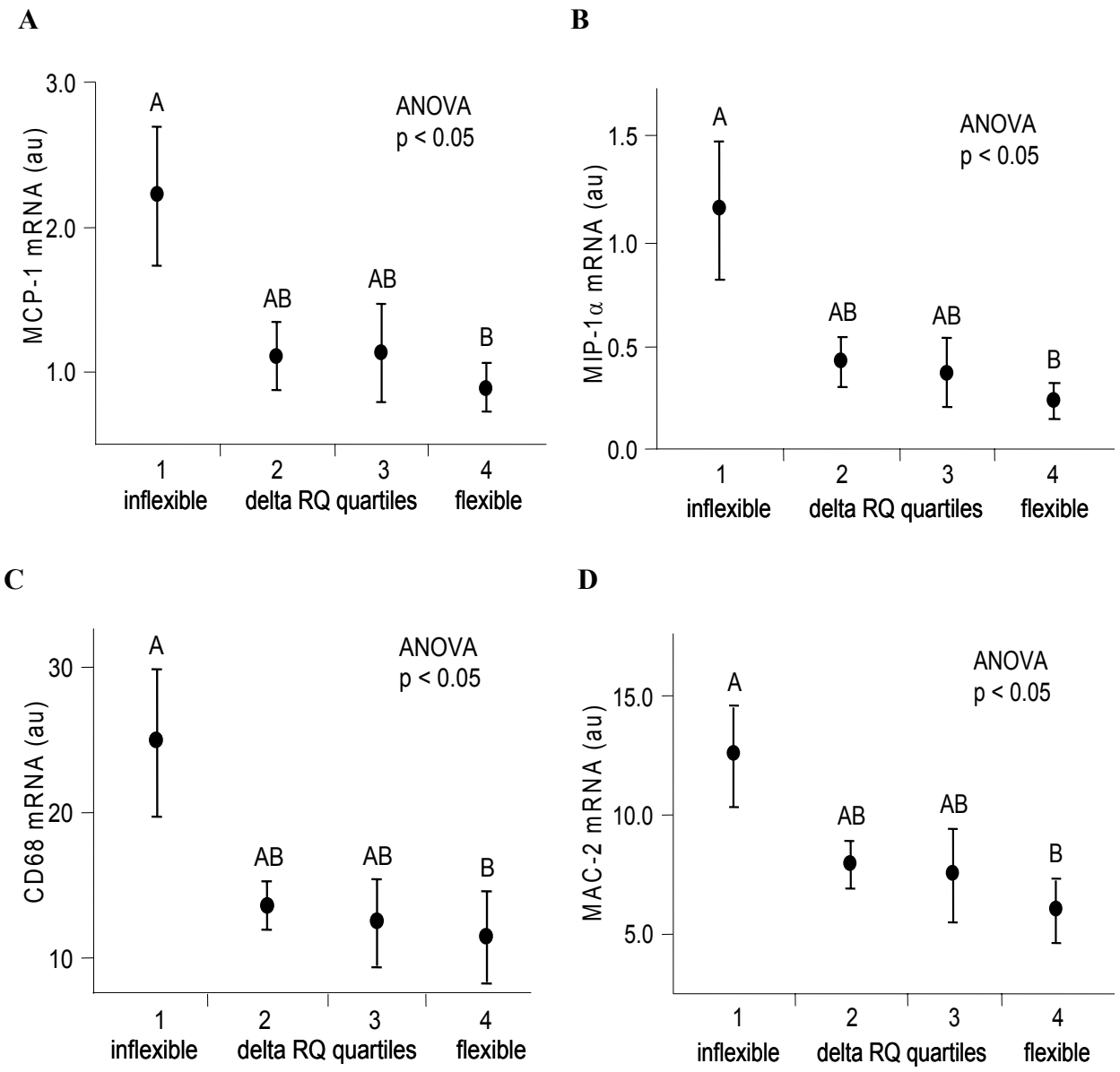
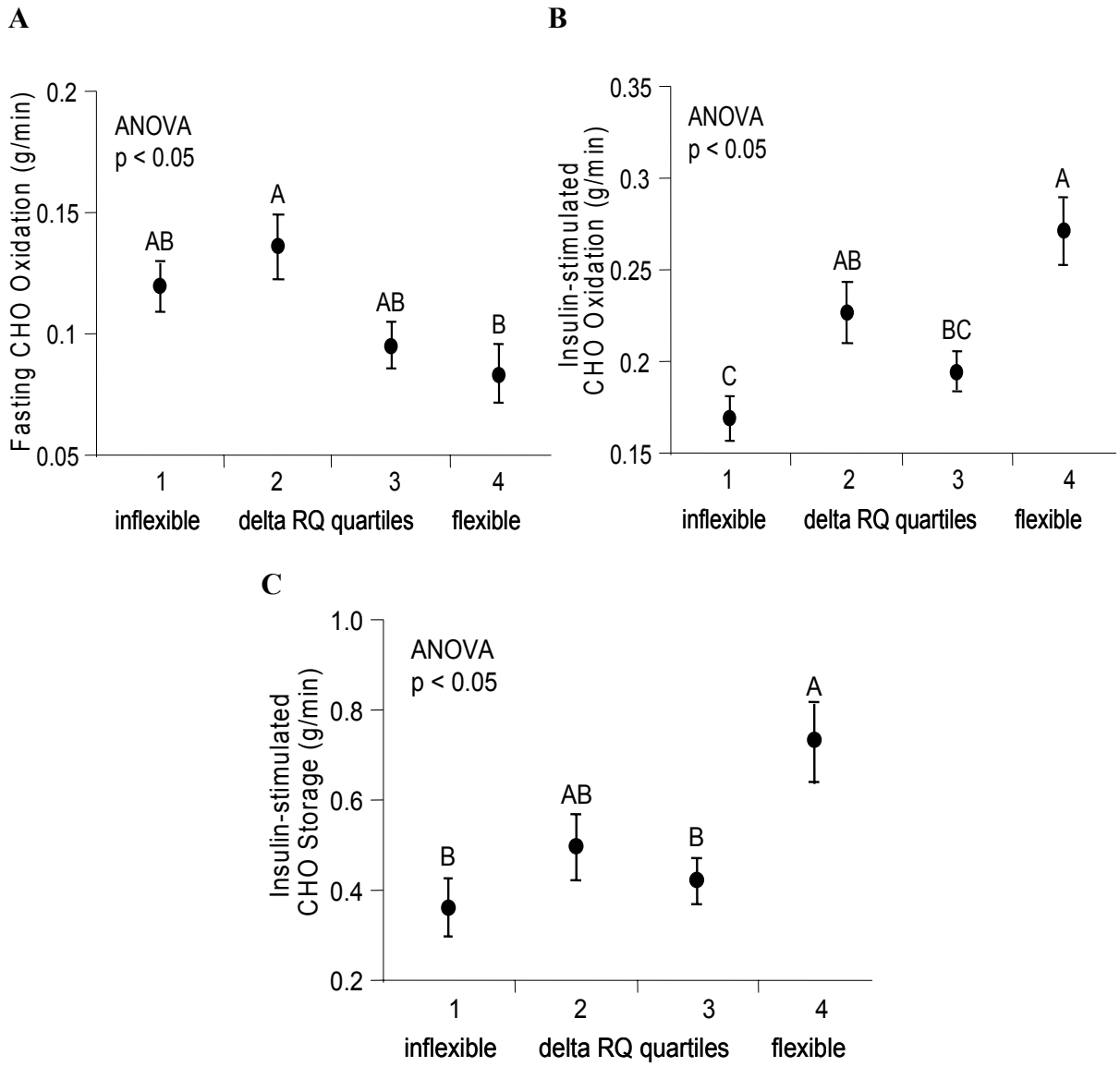


Figure 5.2 Relationships between metabolic inflexibility (ΔRQ) and expressions of chemokines and macrophage markers. The change in respiratory quotient (ΔRQ) was divided into quartiles and correlated with gene expressions of chemokines and macrophage markers in adipose tissue of 56 young healthy men. Both MCP-1 expression (A) and MIP-1α expression (B) were negatively correlated with ΔRQ. Both CD68 expression (C) and MAC-2 expression (D) were also negatively correlated with ΔRQ. mRNA expression data were normalized to 18S. Delta RQ (ΔRQ) was subdivided into quartiles. Data are shown as means ± SE. All pairs were compared using Tukey-Kramer HSD, alpha = 0.05. Levels not connected by same letter are significantly different.

Figure 5.3 Oxidative and non-oxidative carbohydrate (CHO) disposal are related to metabolic inflexibility (ΔRQ) in healthy young men. Higher fasting carbohydrate (CHO) oxidation (A) was associated with lower ΔRQ. Lower levels of insulin-suppressed CHO oxidation (B) and storage (C) were associated with lower ΔRQ. Delta RQ (ΔRQ) was subdivided into quartiles. ANOVA was used to test for differences in biopsy and blood parameters across quartiles of metabolic flexibility (ΔRQ), with post-hoc testing by mean equality contrast between different groups using the Tukey-Kramer HSD; alpha = 0.05. Type I error rate was set a priori at $p < 0.05$. Data are shown as means $\pm$ SE. All Levels not connected by same letter are significantly different.
were positively correlated with CD68 and MAC-2 (Table 5.2).


A
Fasting CHO Oxidation (g/min)
0.2
0.15
0.1
0.05
ANOVA
p < 0.05
AB
A
AB
B
1
2
3
4
inflexible
delta RQ quartiles
flexible
B
Insulin-stimulated
CHO Oxidation (g/min)
0.35
0.3
0.25
0.2
0.15
ANOVA
p < 0.05
C
AB
BC
A
1
2
3
4
inflexible
delta RQ quartiles
flexible
C
Insulin-stimulated
CHO Storage (g/min)
1.0
0.8
0.6
0.4
0.2
ANOVA
p < 0.05
B
AB
B
A
1
2
3
4
inflexible
delta RQ quartiles
flexible

correlated with MCP-1 and MIP-1α (Table 5.2). There was no relationship between the macrophage markers CD68 and MAC-2 and fasting FFAs. Insulin-suppressed FFAs

We divided metabolic flexibility (ΔRQ) into quartiles (quartile 1 = ΔRQ < 0.06; quartile 2 = 0.06 < ΔRQ ≤ 0.08; quartile 3 = 0.08 < ΔRQ ≤ 0.11; quartile 4 = ΔRQ > 0.11) and compared quartiles to adipose tissue inflammatory gene expression. The chemokines MCP-1 and MIP-1α were higher in inflexible subjects (lower ΔRQ) (ANOVA, $p < 0.05$; Figure 5.2.A and ANOVA, $p < 0.05$; Figure 5.2.B, respectively), as were the macrophage markers CD68 and MAC-2 (ANOVA, $p < 0.05$; Figure 5.2.C and ANOVA, $p < 0.05$; Figure 5.2.D).

Metabolic inflexibility is described as both higher fasting glucose oxidation (high RQ) and lower insulin-stimulated glucose oxidation (low RQ). To explore the contribution of these two possibilities, we calculated fasting glucose oxidation, as well as oxidative and non-oxidative glucose disposal. Lower ΔRQ (metabolic inflexibility) was associated with higher fasting carbohydrate (CHO) oxidation (ANOVA, $p < 0.05$; Figure 5.3.A), while lower insulin-suppressed CHO oxidation was associated with lower ΔRQ (ANOVA, $p < 0.05$; Figure 5.3.B). Lower non-oxidative CHO disposal (CHO storage) was associated with lower ΔRQ (ANOVA, $p < 0.05$; Figure 5.3.C). Upon insulin stimulation, CHO storage was significantly greater than CHO oxidation for both flexible (higher ΔRQ) and inflexible subjects (lower ΔRQ) (0.57 ± 0.06 vs. 0.22 ± 0.02 g/min and 0.43 ± 0.05 vs. 0.20 ± 0.01 g/min, respectively; data not shown).

5.3 Discussion

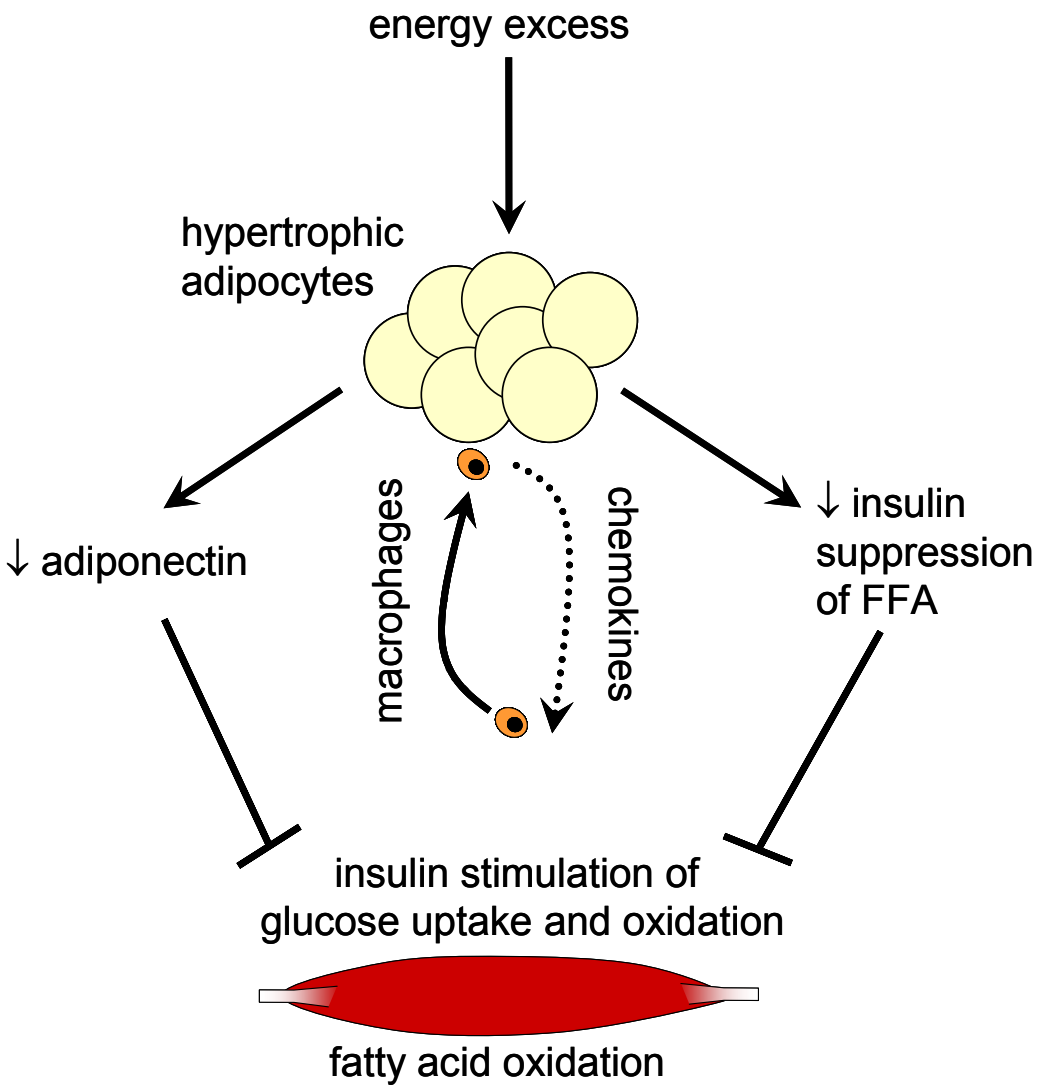
Increased body fatness, coupled with adipocyte hypertrophy, is a determinant of metabolic inflexibility. While metabolic flexibility varied greatly (0.03 – 0.25) across the

cohort of 56 healthy young males, those with a higher percentage of body fat and larger fat cells were more inflexible (lower ΔRQ). Obesity and hypertrophic adipocytes are associated with increased free fatty acids (FFAs) and insulin-resistant adipocytes. In the present study, lower serum adiponectin levels were associated with metabolic inflexibility. Fat cell size (FCS) was positively correlated with fasting and insulin-suppressed FFAs, which are essentially free fatty acid levels upon insulin stimulation during steady state of a euglycemic-hyperinsulinemic clamp (EHC); however, metabolic inflexibility was not related to fasting FFAs. In fact, the novel finding we describe is a relationship between metabolic inflexibility and insulin-suppressed FFAs.

Adipocyte hypertrophy and metabolic inflexibility are also related to disordered lipid metabolism. Our results revealed that higher insulin-suppressed FFAs were associated with higher gene expressions of certain macrophage markers (CD68 and MAC-2) and chemokines (MCP-1 and MIP-1α); however, fasting FFAs were not related to any of the inflammatory gene expressions. Fat cell size was positively correlated with the macrophage CD68 and the chemokine MIP-1α. The expressions of these mRNAs suggest a presence of macrophages and chemokines, as in the inflammatory state. Furthermore, their increased expression as insulin-suppressed free fatty levels increase supports a relationship between macrophage infiltration and chemotaxis and a reduced ability of the adipose tissue to respond to insulin's signal of lipolysis suppression. Even more striking was that mRNA expression of both the macrophage markers and the chemokines coordinately increased, suggesting an interdependent, and perhaps causal, relationship between the two groups. Our results also revealed that metabolically inflexible subjects had higher gene expressions of both macrophage markers (CD68 and

Figure 5.4 Metabolic flexibility was affected by body fatness, adipocyte hypertrophy, chemokines and macrophage content and adiponectin. Energy excess leads to increased body fatness (%) and hypertrophic adipocytes. Hypertrophic adipocytes secrete chemokines and lead to macrophage infiltration. The macrophage-infiltrated hypertrophic adipocytes decrease insulin stimulated suppression of lipolysis, as well as decrease adiponectin secretion. Elevated levels of FFAs during steady state (insulin infusion), coupled with decreased serum adiponectin levels, lead to an inhibition of insulin-stimulated glucose uptake and oxidation in skeletal muscle and a decreased capacity for fat oxidation.

Figure 5.4


energy excess
hypertrophic
adipocytes
↓ adiponectin
macrophages
chemokines
↓ insulin
suppression
of FFA
insulin stimulation of
glucose uptake and oxidation
fatty acid oxidation

MAC-2), as well as both chemokines (MCP-1 and MIP-1α). These relationships implicate adipocyte hypertrophy, increased chemokines and macrophage infiltration as causes of an impaired insulin suppression of FFAs and metabolic inflexibility. The expression of genes involved in chemotaxis and macrophage content are higher in obesity and related metabolic disorders (144-149). Metabolic inflexibility is classically described as both higher fasting glucose oxidation (high RQ) and lower insulin-stimulated glucose oxidation (low RQ). Metabolically inflexible subjects had higher fasting carbohydrate (CHO) oxidation and both lower insulin-stimulated CHO oxidation and storage.

Metabolic flexibility is affected by body fatness, adipocyte hypertrophy, chemokines and macrophage content. This data is consistent with a model where energy excess increases body fat and adipocytes become hypertrophic. Hypertrophic adipocytes secrete chemokines, such as MCP-1, that recruit macrophages, leading to macrophage infiltration of adipose tissue. The macrophage-infiltrated hypertrophic adipocytes secrete cytokines that decrease adipocyte insulin sensitivity leading to impaired suppression of FFAs. Taken together, these disturbances in adipocyte functioning lead to decreased insulin-stimulated glucose uptake and oxidation, as well as a decreased capacity for fat oxidation in skeletal muscle (Figure 5.4). This model and experimental data are consistent with a growing body of literature.

For example, FFAs suppress insulin-stimulated glucose disposal. Boden et al showed that FFA infusion during a clamp leads to proportional inhibitions of insulin-stimulated glucose uptake and of intracellular glucose utilization (150). Heilbronn et al has shown that administration of recombinant adiponectin in rodents increases glucose uptake and fat oxidation in muscle and improves whole body insulin resistance (151).

Additionally, recent studies by Civitarese et al demonstrated that low adiponectin levels are associated with low mitochondrial DNA (mtDNA) copy number, as well as decreased mitochondrial functions (152).

To date, the accepted idea has emerged that chronic activation of the inflammatory pathway can be a mechanism for certain obesity-related metabolic disorders, such as Type 2 diabetes (28). Consequently, while there is no question that the inflammatory pathway becomes activated in adipose tissue in various obese states, and that this negatively impacts insulin action in adipocytes, the total contribution of adipose tissue as a source of these cytokines and/or chemokines is still unclear. In this respect, recent attention has focused on the potential role of macrophages in this process (153). Weisberg et al. and Xu et al. have shown that in obesity, adipose tissue contains an increased number of resident macrophages and that, in some circumstances, macrophages can constitute up to almost half of the cell population within an adipose tissue depot (22; 23). Macrophages have been shown to be a potential source of secreted proinflammatory factors, and these data have led to the concept that macrophages can directly influence adipocyte biology and ultimately lead to a state of insulin resistance. Immunohistochemical analysis from Weisberg et al. of human subcutaneous adipose tissue revealed that the percentage of cells expressing the macrophage antigen CD68 was significantly and positively correlated with both adipocyte size and body mass (22).

Enlarged adipocytes are associated with metabolic inflexibility and are an independent predictor of Type 2 diabetes. To understand the molecular link between these diseases and adipocyte hypertrophy, Jernas, et al. developed a technique to separate human adipocytes from an adipose tissue sample into populations of small cells and large

cells. They identified genes with markedly higher expression in large, compared with small, human adipocytes, and they concluded that these genes may link hypertrophic obesity to Type 2 diabetes (154). This transcriptional link between adipocyte hypertrophy and insulin resistance is complementary to our data showing a physiological link between adipocyte hypertrophy and impaired insulin suppression of lipolysis, which leads to metabolic inflexibility. The risk of metabolic complication is increased not only by the amount and location of adipose tissue, but also by the size of the fat cells. Human fat cells can change ~20-fold in diameter and several thousand-fold in volume. Lipid mobilization and glucose metabolism are increased in enlarged adipocytes (155). In contrast, the stimulating effect of insulin on the rate of glucose metabolism is inversely related to the size of the fat cell (156; 157). Cytokine release within adipose tissue also appears to be correlated with adipocyte size (158-161), and hypertrophic adipocytes may contribute to lipotoxicity (162). Thus, adipocyte hypertrophy leads to macrophage infiltration and chemotaxis, ultimately resulting in an impaired insulin suppression of free fatty acids.

FFA levels are pivotal in complex disease states such as T2D, as well as those that precede it, such as metabolic inflexibility. Recent studies by Bajaj et al. (163) demonstrate an improvement in peripheral insulin action in response to a 7-day reduction of plasma FFAs in Type 2 diabetics. Experiments by Hickner et al. (164) in which they used a microdialysis technique to determine suppression of whole body and regional lipolysis by insulin in sedentary pre-menopausal women revealed a resistance to the suppression of lipolysis by insulin in obesity. Correlated with intraabdominal fat mass,

this impaired insulin suppression of lipolysis leads to an increase in FFA availability primarily in the liver and muscle, as well as other organs.

Past studies have shown that fat infusions (163) inhibit carbohydrate metabolism. An impaired insulin suppression of lipolysis leads to excess circulating free fatty acids. Accumulation of excess fatty acids in the form of triacylglycerol (TAG) in skeletal muscle and in liver is associated with metabolic inflexibility (70; 165; 166). This increase in fat flux leads to 'metabolic inflexibility' whereby the skeletal muscle is unable to switch substrates from fat to carbohydrate. The link between adipose tissue and metabolic flexibility emphasizes the important role of adipose tissue in buffering the daily influx of dietary fat entering the circulation and preventing excessive exposure of other tissues to this fat influx.

Body fatness, chemotaxis, macrophage infiltration and impaired insulin suppression of FFAs combine to contribute to a diminished capacity of substrate switching from that of fat oxidation to carbohydrate oxidation as measured by the change in respiratory quotient when infused with insulin. Currently, the literature points the finger at inflammation, particularly macrophage infiltration (167-172), as the culprit in obesity that eventually leads to metabolic disorders such as Type 2 diabetes. However, to date no one has determined the cause. Our study has implicated a middle man between metabolic disorders related to obesity, such as metabolic inflexibility and the state of inflammation—blunted insulin suppression of lipolysis, which stems from adipocyte hypertrophy.

CHAPTER 6: SUMMARY AND CONCLUSIONS

6.1 Summary

Studies have suggested that defects in the ability to switch substrate oxidation from fat to carbohydrate during insulin stimulation might contribute to development of obesity and/or the metabolic sequelae of obesity (173). Metabolism involves the adaptation to both the supply and demand of energy, and this adaptability requires 'a clear capacity to utilize lipid and carbohydrate fuels and to transition between them' (123). In the healthy state, this transitioning between substrates is termed 'metabolic flexibility'. However, as it always is according to the laws of the universe, this state of flexibility has a counterpart characterized by the body's inability to switch substrate utilization under appropriate conditions. This state is termed 'metabolic inflexibility'. Increasing evidence points to metabolic inflexibility as a key dysfunction of the cluster of disease states that contribute to the metabolic syndrome, such as Type 2 diabetes. Two main features of metabolic inflexibility are: (1) failure of skeletal muscle to appropriately switch between the use of lipid in the fasting state and use of carbohydrate upon insulin stimulation and (2) an impaired transition from fatty acid efflux to storage in response to a meal. The reduced ability to metabolize fuel, such as oxidative phosphorylation in the electron transport chain in skeletal muscle, is indicated by reduced mitochondrial size and function, and it is characteristic of metabolic inflexibility (70; 123).

Our studies on the effects of an acute high fat diet (HFD) in a population of young healthy males and mice demonstrated that substrate switching in skeletal muscle occurs via classic endocrine/physiologic systems, as well as at the transcriptional level.

Importantly, there were no changes in fasting glucose, insulin and free fatty acids after the diet intervention, fatty acid oxidation did increase as measured by a decrease in respiratory quotient (RQ). Using oligonucleotide microarray technology, we found that about 300 genes were regulated by the three-day high fat diet in the skeletal muscle of healthy young men. The first part of the microarray analysis, along with quantitative real time RT-PCR, revealed a downregulation of OXPHOS genes after the HFD, as well as transcription factors and cofactors involved in mitochondrial biogenesis. Additionally, we observed that the reductions in the expression of genes involved in oxidative phosphorylation and mitochondrial biogenesis were recapitulated in an animal model of dietary-induced obesity and insulin resistance (134) and were of a much greater magnitude in mice as compared to man.

In the second part of the microarray analysis, we expanded the investigation of the effects of an acute high fat/low carbohydrate diet (HF/LCD) to the transcriptional responses for other metabolic systems such as carbohydrate oxidation and storage, as well as fatty acid oxidation. Muscle cells adjust the fuels they oxidize and match substrate supply to demand. Fuel selection shifts toward the oxidation of fatty acids and reduces the oxidation of glucose at the transcriptional level. A positive carbohydrate balance was observed at the end of the three-day HF/LCD in the cohort of the healthy young males, indicating a decrease in glucose oxidation. We also observed a decrease in the messenger RNA levels of a number of genes involved in the oxidative, as well as non-oxidative, glucose metabolism. Furthermore, the analogous murine experiment showed a similar effect (albeit with a greater magnitude of change).

Increased free fatty acid (FFA) levels are pivotal in complex disease states such as Type 2 diabetes (T2D). It has recently been shown in obesity and other complex disease states that there exists a resistance to suppression of lipolysis by insulin. This trait is correlated with intraabdominal fat mass; furthermore, the impaired insulin suppression of lipolysis leads to an increase in FFA availability at the liver and muscle. Leanness, insulin sensitivity and fat cell size determine metabolic flexibility, and are subsequently related to fasting free fatty acid (FFA) levels, as well as FFAs upon insulin infusion. Our experiments revealed impaired insulin suppression of free fatty acids in a cohort of healthy young men during a euglycemic-hyperinsulinemic clamp (EHC). Fat cell size was positively correlated with fasting and insulin-suppressed FFAs, as well as expression of macrophage markers and chemokines. Gene expression of macrophage markers (CD68 and MAC-2) and chemokines (MCP-1 and MIP-1α) was positively correlated with insulin-suppressed FFAs. Even more striking was that the expression of both the macrophage markers and the chemokines was coordinately increased. One novel aspect of this work was the negative correlation between insulin-suppressed FFAs and glucose disposal rate (GDR), as well as fat cell size, chemokines and markers of macrophage content. Interestingly, we observed a consistent negative correlation between "metabolic flexibility" as measured by the change in respiratory quotient (RQ) and several adipose tissue parameters such as percent body fat, fat cell size, insulin suppression of FFAs and expression of chemokines and macrophage markers. Not only did we demonstrate the expected relationships among adipocyte hypertrophy and glucose disposal, but we revealed a consequence of adipocyte hypertrophy, increased expression of macrophage markers and chemokines and subsequent impaired insulin suppression of lipolysis.

6.2 Conclusions

The link between obesity and insulin resistance has been recognized for many years. In the past several years, evidence that a disturbance in muscle lipid oxidative capacity results in the accumulation of the lipid intermediates that interfere with insulin signaling and ultimately inhibit insulin-mediated glucose uptake and subsequent metabolism. Underlying mechanisms for disturbed fatty acid handling may relate to impaired adipose tissue lipolysis and a reduced ability to oxidize fat. Many of these abnormalities have been reported both in the obese and Type 2 diabetics (174-176). Increased free fatty acid (FFA) flux from adipose tissue to non-adipose tissue is at the heart of many metabolic disturbances characteristic of insulin resistance and Type 2 diabetes.

It has been purported that high fat diets and/or high fat flux through the mitochondria reduce the expression of nuclear genes encoding mitochondrial proteins and transcription factors involved in mitochondrial biogenesis. Our data support this hypothesis. Previous studies suggest a link between the downregulation of PGC1 and the dysregulation of OXPHOS genes. Our results are consistent with this sequence of events, and indeed three of our OXPHOS genes were also present in analyses by Patti and Mootha (83; 84). We also move our view upstream, beyond the relationship between PGC1 and OXPHOS genes. We showed an increased fatty acid flux through the mitochondria decreases PGC1 expression and is associated with a downregulation of expression of genes involved in oxidative phosphorylation. Our studies support the novel hypothesis that high fat diets explain the reduction in oxidative capacity that is seen in aging, obesity, insulin resistance and Type 2 diabetes.

The acute effects of fatty acids on glucose metabolism and storage have been shown enzymatically; however, our studies supplement these effects by demonstrating a downregulation of genes involved in glucose oxidation and storage when fat intake is increased and carbohydrate intake is decreased. Therefore, we expand the view of the 'glucose-fatty acid cycle' beyond enzymatic activity to the transcriptional level. Overall, our identification of multiple regulated genes within the same pathway by a high fat/low carbohydrate diet is novel.

Adipose tissue in obesity becomes noncompliant to suppression of fat mobilization, i.e. lipolysis, by insulin, and also to the normal insulin-stimulated fat storage by activation of lipoprotein lipase. The net effect is "full" adipocytes that are unable to store more fat; thus, in the post-prandial state after a meal, there is an excess flux of lipid that would normally have been absorbed by the adipose tissue. This situation leads to fat deposition in other tissues. Accumulation of triacylglycerol in skeletal muscle and in liver is associated with insulin resistance. Furthermore, this increase in fat flux leads to 'metabolic inflexibility' in whereby the skeletal muscle is unable to switch substrates from fat to carbohydrate. The link between adipose tissue and insulin resistance and metabolic flexibility emphasizes the important role of adipose tissue in buffering the daily influx of dietary fat entering the circulation and preventing excessive exposure of other tissues to this influx. Our studies have demonstrated a physiological relationship among adipocyte hypertrophy, impaired insulin suppression of adipose tissue lipolysis and glucose disposal in skeletal muscle. We also revealed at the transcriptional level an increase in macrophage markers and chemokines. These data

suggest an infiltration of macrophages and chemotaxis leads to the state of inflammation present in obesity and its related metabolic disorders, such as metabolic inflexibility.

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APPENDIX A: SUPPLEMENTAL TABLES

Gene	Accession number	Forward primer	Probe	Reverse primer
NDUFB3	NM_002491	TCAGATTGCTGTCAGACATGG	Sybr Green I detection	TGGTGTCCCTTCTATCTTCCA
NDUFB5	NM_002492	TTCGACACAGTGGAGACCAT	Sybr Green I detection	TTCAGCTTGACCAATGAATACA
NDUFV1	NM_007103	CTGATCCCCAAGTCTGTGTGTGAGA	Sybr Green I detection	GATGTCCGTCGAGCGGTCCAT
NDUFS1	NM_005006	GAATGTGATCTGCAGGACCAGTC	Sybr Green I detection	GTCTTTACCAATGGCCCAATGT
SDHB	NM_003000	AAATGTGGCCCCATGGTATTG	Sybr Green I detection	AGAGCCACAGATGCCTTCTCTG
SLC25A12	NM_003705	AGCACTCTAGCTGGCACAAGGAA	Sybr Green I detection	AGTCAAGCGCCCTGAAGCATTATAT
18S	NM_000979	GATATGCTCATGTGGTGTTGA	Sybr Green I detection	ACGTTCCACCTCATCCTCA
CYC1	NM_001916	TGGCCCCTCCCATCTACAC	AGAGTTTGACGATGGCACCCCAGCTA	ATCCTTGGCTATCTGGGACATG
SURF1	NM_003172	CCTATGTGGTCACTCCCTTCCA	TGGTGACTCCCAGGTCGGTGCA	TGGGAACGAACCCTCTATTTACC
PGC1α	NM_015062	AGGTGAAAGTGTAATACTGTTGGTTGA	TGCTGAAGAGGGAAAGTGAGCGATTAGTTGA	CATGTAGAATTGGCAGGTGGAA
PGC1β	NM_133263	CAGCCACTCGAAGGAACTTCA	CTGAACACGGCCCTCTGCTCTCACA	CGGATGCTTGGCGTTCTG
TFAM	NM_009360	CCCAGATGCAAAAACTACAGAACTAA	CGCAGTCACAACACTTACAAGCCAAAC	TCCGCCCTATAAGCATCTTGA
NRF1	NM_005011	CGTTGCCCAAGTGAATTATTCTG	TTGTTCCACCTCTCCATCAGCCA	CCCTGTAACGTGGCCCAAT
RPLP0	NM_001002	CCATTCTATCATCAACGGGTACAA	TCTCCACAGACAAGGCCAGGACTCG	AGCAAGTGGGAAGGTGTAATCC
mmNDUFB3	NM_025597	TGGCCATGGTAAAATGGAACT	CAGATTACAGACAGTGGAAAATTGAAGGGACG	AAGCTTCTTCTGCACCGTTTCT
mmNDUFB5	NM_025316	GCATCCGATATCAAGATGGATCG	CTGAAAAGAACTACGAGAAAACCCTGGCTATCCT	CTTTAACCGTAACTCAGCCTTTTCA
mmSLC25A12	NM_172436	CACCACAGGACCGAGAGTCA	TGCTTCAGGACCTGGGACTTTTCGG	GGCTTTGGCACCCTTGTACA
mmSDHB	NM_023374	CCCAGGAGGGCAAGCAA	AGTATCTGCAGTCCATCGAGGACCGG	GTACAGCCCGTCCAGCTTCT
mmCYC1	NM_025567	CAGCTACCCATGGTCTCATCGT	TCCGAATGCTGGTGTGGTCCAAGGA	CACCTGCTTGTATACCTGGAAACC
mmSURF1	NM_013677	AAGTGAATCCTGAGACCAGACAGA	AGGCCAGGTTCTGGGAGAAGTAGACC	TGTGAGCCTCACTATGCCAACT
mmPGC1α	NM_008904	CATTTGATGCACTGACAGATGGA	CCGTGACCACTGACAACGAGGCC	CCGTCAGGCATGGAGGAA
mmPGC1β	NM_133249	AGGAAGCGGCGGGAAA	AGAGATTTCGAATGTATACCACACGGCCTTCA	CTACAATCTCACCGAACACCTCAA
mmCOXII	NC_005089	TTTTCAGGCTTCACCCTAGATGA	CATGAGCAAAAGCCCACTTCGCCA	GAAGAATGTTATGTTTACTCCTACGAATATG
mmUCP2	NM_011671	GCGTTCTGGGTACCATCCTAAC	CGCACTGAGGGTCCACGCAGC	GCGACCAGCCCATTGTAGA
mmCycB	NM_011149	GGTGGAGAGCACCAAGACAGA	ATCCTTCAGTGGCTTGTCCCGGCT	GCCGGAGTCGACAATGATG

A.1 Oligonucleotide sequences for primer/probe sets used for qRT-PCR. For all assays performed using SYBR Green I detection, 18S was used as the internal control, and for all assays performed using Taqman primers and probe, RPLP0, which is the human equivalent of the murine gene 36B4, was used as the internal control. Cyclophilin B was used as the internal control for all murine assays. NDUFB3, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3; NDUFB5, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5; NDUFV1, NADH dehydrogenase (ubiquinone) flavoprotein 1; NDUFS1, NADH dehydrogenase (ubiquinone) Fe-S protein 1); SDHB, succinate dehydrogenase complex, subunit B; SLC25A12, solute carrier family 25 (mitochondrial carrier); 18S, 18S ribosomal RNA; CYC1, cytochrome c-1; SURF1, surfeit 1; PGC1α, peroxisome proliferative activated receptor, gamma,

coactivator 1, alpha; PGC1β, peroxisome proliferative activated receptor, gamma, coactivator 1, beta; TFAM, transcription factor A, mitochondrial; NRF1, nuclear respiratory factor-1; RPLP0, ribosomal protein, large, P0; mm, mus musculus; mmNDUFB3, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3; mmNDUFB5, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5; mmSLC25A12, solute carrier family 25 (mitochondrial carrier); mmSDHB, succinate dehydrogenase complex, subunit B; mmCYC1, cytochrome c-1; mmSURF1, surfeit 1; mmPGC1α, peroxisome proliferative activated receptor, gamma, coactivator 1, alpha; mmPGC1β, peroxisome proliferative activated receptor, gamma, coactivator 1, beta; mmCOXII, cytochrome c oxidase II, mitochondrial; mmUCP2, uncoupling protein 2; mmCycB, peptidylprolyl isomerase B (cyclophilin B).

CLUSTER	ACCESSION	GENE DESCRIPTION	FOLD CHANGE	DIRECTION	FUNCTION	BONFERONNI P-VALUE
1	AB028968	Homo sapiens mRNA for KIAA1045 protein, partial cds	1.5	down	biological process unknown	0.002999056
1	AB037743	Homo sapiens mRNA for KIAA1322 protein, partial cds	1.4	down	developmental processes	0.003082866
1	AB039903	Homo sapiens ifp1 mRNA for interferon-responsive finger protein 1 long form, complete cds	2	down	pathogenesis	0.000120886
1	AB046796	Homo sapiens mRNA for KIAA1576 protein, partial cds	1.4	down	ethanol oxidation	0.000091228
1	AF052087	Homo sapiens clone 23604 mRNA sequence	1.1	down	DNA packaging	3.34674E-06
1	AF060169	Homo sapiens AS11 protein mRNA, partial cds	1.8	down	transcription regulation	0.001436171
1	AF086156	Homo sapiens full length insert cDNA clone ZB55H12	1.1	down	biological process unknown	0.002373667
1	AF086350	Homo sapiens full length insert cDNA clone ZD62G08	2.2	down	biological process unknown	0.008001914
1	AF090102	Homo sapiens clone IMAGE 21785	2.1	down	biological process unknown	0.002811766
1	AK000032	Homo sapiens cDNA FLJ20025 fis, clone ADSE01840	1.8	down	biological process unknown	0.00025246
1	AK000789	Homo sapiens cDNA FLJ20782 fis, clone COL03841	2.6	down	biological process unknown	1.42117E-09
1	AK021462	Homo sapiens cDNA FLJ11400 fis, clone HEMBA1000673	2.4	down	biological process unknown	1.37972E-05
1	AK022297	Homo sapiens cDNA FLJ12235 fis, clone MAMMA1001243	1.7	down	biological process unknown	0.000513922
1	AK026037	Homo sapiens cDNA: FLJ22384 fis, clone HRC07594	1.4	down	biological process unknown	0.004993469
1	AL117653	Homo sapiens mRNA; cDNA DKFZp586C0224 (from clone DKFZp586C0224)	2.2	down	biological process unknown	0.000065843
1	AL137363	Homo sapiens mRNA; cDNA DKFZp434D1026 (from clone DKFZp434D1026)	1.4	down	biological process unknown	0.003665174
1	AL137597	Homo sapiens mRNA; cDNA DKFZp434B2411 (from clone DKFZp434B2411)	2.2	down	biological process unknown	0.00010901
1	AL157476	Homo sapiens mRNA; cDNA DKFZp761C082 (from clone DKFZp761C082)	1.7	down	cell adhesion	0.008059978
1	D87292	Homo sapiens mRNA for rhodanese, complete cds	1.9	down	cyanate catabolism	0.00610911
1	L19362	Human (clone xip1) mRNA sequence	1.5	down	biological process unknown	0.000444252
1	NM_000155	Homo sapiens galactose-1-phosphate uridylyltransferase (GALT) mRNA	1.6	down	galactose metabolism	0.009046843
1	NM_000476	Homo sapiens adenylate kinase 1 (AK1), nuclear gene encoding mitochondrial protein, mRNA	1.5	down	nucleic acid metabolism	0.000990824
1	NM_000532	H.sapiens propionyl Coenzyme A carboxylase, beta polypeptide (PCCB), mRNA	1.6	down	fatty acid catabolism	2.98029E-06
1	NM_000666	Homo sapiens aminoacylase 1 (ACY1), mRNA	1.6	down	proteolysis and peptidolysis	0.000398322
1	NM_001297	Homo sapiens cyclic nucleotide gated channel beta 1 (CNGB1) mRNA, and translated products	1.7	down	vision	2.01462E-09
1	NM_001412	Homo sapiens eukaryotic translation initiation factor 1A (EIF1A) mRNA	2	down	protein synthesis initiation	0.001449064
1	NM_002487	Homo sapiens necdin (mouse) homolog (NDN) mRNA	1.2	down	neurogenesis	0.001234221
1	NM_002491	Homo sapiens NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3 (12kD, B12) (NDUFB3) mRNA	1.4	down	complex I (NADH to ubiquinone)	0.007275987
1	NM_003974	Homo sapiens docking protein 2, 56kD (DOK2) mRNA	1.4	down	signal transduction (cell surface receptor)	0.005136911
1	NM_004236	Homo sapiens thyroid receptor interacting protein 15 (TRIP15) mRNA	1.9	down	signal transduction	0.00960494
1	NM_007126	Homo sapiens valosin-containing protein (VCP), mRNA	2	down	intracellular protein traffic	0.000413305
1	NM_014056	Homo sapiens DKFZP564K247 protein (DKFZP564K247), mRNA	1.7	down	mitochondrial processing	1.14425E-07
1	NM_016417	Homo sapiens clone FLB4739 (LOC51218), mRNA	1.5	down	biological process unknown	0.001473864
1	NM_016599	Homo sapiens muscle-specific protein (LOC51778), mRNA	2.1	down	biological process unknown	0.000000
1	X78938	H.sapiens SRI mRNA	1.6	down	biological process unknown	0.00688159
1	AL049471	Homo sapiens mRNA; cDNA DKFZp586N012 (from clone DKFZp586N012)	1.7	up	biological process unknown	2.22666E-06
1	L08902	Homo sapiens mRNA fragment	1.8	up	humoral defense mechanism	9.4576E-08
1	L09070	Homo sapiens mRNA fragment	1.8	up	blood coagulation	9.4576E-08
1	L09071	Homo sapiens mRNA fragment	1.8	up	blood coagulation	9.4576E-08
1	L09078	Homo sapiens mRNA fragment	1.8	up	humoral defense mechanism	9.4576E-08
1	L09080	Homo sapiens mRNA fragment	1.8	up	blood coagulation	9.4576E-08
1	L09081	Homo sapiens mRNA fragment	1.8	up	blood coagulation	9.4576E-08
1	L09086	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08
1	L09089	Homo sapiens mRNA fragment	1.8	up	humoral defense mechanism	9.4576E-08
1	L09092	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08
1	L09095	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08
1	L09097	Homo sapiens mRNA fragment	1.8	up	proteolysis and peptidolysis	9.4576E-08
1	L09098	Homo sapiens mRNA fragment	1.8	up	humoral defense mechanism	9.4576E-08
1	L09099	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08
1	L09100	Homo sapiens mRNA fragment	1.8	up	blood coagulation	9.4576E-08
1	L09102	Homo sapiens mRNA fragment	1.8	up	blood coagulation	9.4576E-08
1	L10140	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08
1	L10141	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08

1	L10146	Homo sapiens mRNA fragment	1.8	up	blood coagulation	9.4576E-08
1	L10147	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08
1	L10148	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08
1	L10151	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	9.4576E-08
1	L23867	Homo sapiens mRNA fragment	1.8	up	biological process unknown	9.4576E-08
1	L23869	Homo sapiens mRNA fragment	1.8	up	biological process unknown	9.4576E-08
1	L23870	Homo sapiens mRNA fragment	1.8	up	biological process unknown	9.4576E-08
1	L43345	Homo sapiens mRNA fragment	1.8	up	biological process unknown	9.4576E-08
1	L43347	Homo sapiens mRNA fragment	1.8	up	biological process unknown	9.4576E-08
1	L43349	Homo sapiens mRNA fragment	1.8	up	biological process unknown	9.4576E-08
1	L43350	Homo sapiens mRNA fragment	1.8	up	biological process unknown	9.4576E-08
1	NM_000067	Homo sapiens carbonic anhydrase II (CA2) mRNA	1	up	protein targeting	0.002923499
1	NM_000954	Homo sapiens prostaglandin D2 synthase (21kD, brain) (PTGDS), mRNA	1.9	up	prostaglandin metabolism	4.78094E-06
1	NM_001492	Homo sapiens growth differentiation factor 1 (GDF1), mRNA	1.4	up	membrane targeting	3.26237E-07
1	NM_001963	Homo sapiens epidermal growth factor (beta-urogastrone) (EGF) mRNA	1.7	up	signal transduction	0.001798554
1	NM_003234	Homo sapiens transferrin receptor (p90, CD71) (TFRC) mRNA	1.5	up	iron transport	0.009
1	NM_005655	Homo sapiens TGFB inducible early growth response (TIEG), mRNA	1.9	up	cell proliferation	1.3307E-09
1	NM_016485	Homo sapiens hypothetical protein (HSPC228), mRNA	1.6	up	cell wall integrity	1.50129E-09
1	NM_017659	Homo sapiens hypothetical protein FLJ20084 (FLJ20084), mRNA	1.3	up	protein modification	0.006886306
1	NM_018593	Homo sapiens hypothetical protein PRO0813 (PRO0813), mRNA	1.7	up	biological process unknown	0.000337473
1	U81380	Human interleukin-13 receptor soluble form mRNA, complete cds	1.2	up	signal transduction (cell surface receptor)	0.000180734
1	X04236	Human small cellular 7 SK mRNA	1.2	up	biological process unknown	0.001137583
1	Z36789	H.sapiens (xs138) mRNA, 250bp	1.9	up	blood coagulation	2.33058E-06
2	AF055030	Homo sapiens clone 24538 mRNA sequence	1.5	down	transcription regulation	0.005462657
2	AF070565	Homo sapiens clone 24425 mRNA sequence	2	down	biological process unknown	2.38024E-08
2	AF086264	Homo sapiens full length insert cDNA clone ZD43A10	1.1	down	biological process unknown	0.000105155
2	AF147426	Homo sapiens full length insert cDNA clone YP79H10	1.3	down	biological process unknown	3.36945E-09
2	AK001906	Homo sapiens cDNA FLJ11044 fis, clone PLACE1004451	1.4	down	biological process unknown	0.002560693
2	AK025657	Homo sapiens cDNA: FLJ22004 fis, clone HEP06871	2.1	down	sensory perception	3.78602E-08
2	AL049361	Homo sapiens mRNA; cDNA DKFZp566H243 (from clone DKFZp566H243)	1.6	down	biological process unknown	2.13573E-05
2	AL050148	Homo sapiens mRNA; cDNA DKFZp586G1520 (from clone DKFZp586G1520)	1.6	down	biological process unknown	9.92366E-05
2	AL390169	Homo sapiens mRNA; cDNA DKFZp547D064 (from clone DKFZp547D064)	1.8	down	biological process unknown	0.000091741
2	NM_000083	Homo sapiens chloride channel 1 , skeletal muscle (CLCN1), mRNA	1.8	down	chloride transport	2.58873E-07
2	NM_000717	Homo sapiens carbonic anhydrase IV (CA4), mRNA	1.5	down	protein targeting	0.0061872
2	NM_001829	Homo sapiens chloride channel 3 (CLCN3) mRNA	1.4	down	chloride transport	0.004788336
2	NM_003167	Homo sapiens sulfotransferase family 2A, dehydroepiandrosterone (DHEA) -preferring, member 1 (SULT2A1) mRNA	1.3	down	steroid metabolism	0.008640503
2	NM_003344	Homo sapiens ubiquitin-conjugating enzyme E2H (homologous to yeast UBC8) (UBE2H) mRNA	1.4	down	proteolysis and peptidolysis	6.68648E-06
2	NM_003665	Homo sapiens ficolin (collagen/fibrinogen domain-containing) 3 (Hakata antigen) (FCN3), mRNA	1.6	down	opsinization	0.000932942
2	NM_004170	Homo sapiens solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter), member 1 (SLC1A1), mRNA	1.3	down	glutamate transport	0.000186263
2	NM_014709	Homo sapiens KIAA0570 gene product (KIAA0570), mRNA	1.2	down	spermatogenesis	0.000871745
2	NM_014752	Homo sapiens KIAA0102 gene product (KIAA0102), mRNA	1.2	down	steroid metabolism	0.00045674
2	NM_014845	Homo sapiens KIAA0274 gene product (KIAA0274), mRNA	1.6	down	synaptic vesicle endocytosis	4.30869E-09
2	NM_017781	Homo sapiens hypothetical protein FLJ20359 (FLJ20359), mRNA	1.4	down	electron transport	0.002432844
2	NM_018467	Homo sapiens uncharacterized hematopoietic stem/progenitor cells protein MDS032 (MDS032), mRNA	1.8	down	immune response	0.000685921
2	AB011152	Homo sapiens mRNA for KIAA0580 protein, partial cds	1.4	up	cytoskeleton integrity	2.58635E-05
2	AB018310	Homo sapiens mRNA for KIAA0767 protein, partial cds	1.4	up	steroid metabolism	0.000210553
2	AB033102	Homo sapiens mRNA for KIAA1276 protein, partial cds	1.5	up	muscle contraction	2.87647E-07
2	AF082657	Homo sapiens Era GTPase A protein (HERA-A) mRNA, partial cds	1.5	up	transport	0.00500205
2	AF085351	Homo sapiens ELISC-1 mRNA, partial cds	1.2	up	biological process unknown	7.24137E-09
2	AF291181	Homo sapiens LHX5 protein mRNA, complete cds	1.3	up	transcription regulation	0.00044144
2	AJ224166	Homo sapiens mRNA containing U19H snoRNA; mRNA 1	1.2	up	cell growth and maintenance	5.90463E-05
2	AK022872	Homo sapiens cDNA FLJ12810 fis, clone NT2RP2002464, weakly similar to DNA repair protein PSO2/SNM1	1.6	up	DNA repair	3.07146E-07
2	AK023610	Homo sapiens cDNA FLJ13548 fis, clone PLACE1007068	1.4	up	biological process unknown	0.005040947
2	AK023683	Homo sapiens cDNA FLJ13621 fis, clone PLACE1010954	1.1	up	cytoskeleton integrity	0.002260697
2	AK024927	Homo sapiens cDNA: FLJ21274 fis, clone COL01781	1.5	up	biological process unknown	0.003126244

2	AK026679	Homo sapiens cDNA: FLJ23026 fis, clone LNG01738	2.1	up	biological process unknown	0.005879573
2	AL161991	Homo sapiens mRNA; cDNA DKFZp761C169 (from clone DKFZp761C169); partial cds	1.4	up	biological process unknown	6.62599E-08
2	L08902	Homo sapiens mRNA fragment	1.8	up	humoral defense mechanism	0.001922163
2	L09070	Homo sapiens mRNA fragment	1.8	up	blood coagulation	0.001922163
2	L09071	Homo sapiens mRNA fragment	1.8	up	blood coagulation	0.001922163
2	L09078	Homo sapiens mRNA fragment	1.8	up	humoral defense mechanism	0.001922163
2	L09080	Homo sapiens mRNA fragment	1.8	up	blood coagulation	0.001922163
2	L09081	Homo sapiens mRNA fragment	1.8	up	blood coagulation	0.001922163
2	L09086	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L09089	Homo sapiens mRNA fragment	1.8	up	humoral defense mechanism	0.001922163
2	L09092	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L09095	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L09097	Homo sapiens mRNA fragment	1.8	up	proteolysis and peptidolysis	0.001922163
2	L09098	Homo sapiens mRNA fragment	1.8	up	humoral defense mechanism	0.001922163
2	L09099	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L09100	Homo sapiens mRNA fragment	1.8	up	blood coagulation	0.001922163
2	L09102	Homo sapiens mRNA fragment	1.8	up	blood coagulation	0.001922163
2	L10140	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L10141	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L10146	Homo sapiens mRNA fragment	1.8	up	blood coagulation	0.001922163
2	L10147	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L10148	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L10151	Homo sapiens mRNA fragment	1.8	up	chymotrypsin	0.001922163
2	L23867	Homo sapiens mRNA fragment	1.8	up	biological process unknown	0.001922163
2	L23869	Homo sapiens mRNA fragment	1.8	up	biological process unknown	0.001922163
2	L23870	Homo sapiens mRNA fragment	1.8	up	biological process unknown	0.001922163
2	L25080	Homo sapiens GTP-binding protein (rhoA) mRNA, complete cds	1.6	up	cell growth and maintenance	0.002526602
2	L43345	Homo sapiens mRNA fragment	1.8	up	biological process unknown	0.001922163
2	L43347	Homo sapiens mRNA fragment	1.8	up	biological process unknown	0.001922163
2	L43349	Homo sapiens mRNA fragment	1.8	up	biological process unknown	0.001922163
2	L43350	Homo sapiens mRNA fragment	1.8	up	biological process unknown	0.001922163
2	M20699	Human terminal deoxynucleotidyltransferase gene, exon 7	1.4	up	DNA replication	5.20357E-09
2	M55536	Human glucose transporter pseudogene	1.4	up	biological process unknown	4.658E-12
2	NM_000367	Homo sapiens thiopurine S-methyltransferase (TPMT) mRNA	1.3	up	nucleic acid metabolism	0.000455608
2	NM_001714	Homo sapiens Bicaudal D (Drosophila) homolog 1 (BICD1) mRNA	1.5	up	embryogenesis and morphogenesis	1.56169E-06
2	NM_002864	Homo sapiens pregnancy-zone protein (PZP) mRNA	1.6	up	intracellular protein traffic	0.005691476
2	NM_002907	Homo sapiens RecQ protein-like (DNA helicase Q1-like) (RECQL) mRNA	1.5	up	DNA repair	0.000516067
2	NM_003375	Homo sapiens voltage-dependent anion channel 2 (VDAC2), nuclear gene encoding mitochondrial protein, mRNA	1.5	up	anion transport	2.28422E-06
2	NM_004259	Homo sapiens RecQ protein 5 (RECQ5) mRNA	1.4	up	DNA repair	1.43416E-06
2	NM_005531	Homo sapiens interferon, gamma-inducible protein 16 (IFI16) mRNA	1.7	up	transcription regulation	9.65381E-07
2	NM_006598	Homo sapiens solute carrier family 12 (potassium/chloride transporters), member 7 (SLC12A7), mRNA	2.8	up	transport	0.000156565
2	NM_007016	Homo sapiens protein similar to E.coli yhdg and R. capsulatus nifR3 (PP35), mRNA	1.4	up	`de novo` pyrimidine biosynthesis	0.000204868
2	NM_014590	Homo sapiens endogenous retroviral family W, env(C7), member 1 (syncytin) (ERVWE1), mRNA	1.2	up	embryogenesis and morphogenesis	2.40867E-06
2	NM_014873	Homo sapiens KIAA0205 gene product (KIAA0205), mRNA	1.7	up	phosphatidic acid biosynthesis	0.003616239
2	NM_015975	Homo sapiens neuronal cell death-related protein (LOC51616), mRNA	1.5	up	nucleic acid metabolism	3.86252E-05
2	NM_016360	Homo sapiens clone HQ0477 PRO0477p (LOC51204), mRNA	1.5	up	nucleotide-excision repair	3.44637E-05
2	NM_018183	Homo sapiens hypothetical protein FLJ10701 (FLJ10701), mRNA	1	up	DNA repair	0.003742495
2	NM_020367	Homo sapiens MIB006 (C12orf6), mRNA	1.1	up	cytoskeleton integrity	0.000435746
2	U10511	Human clone 2D12 Cri-du-chat critical region mRNA, partial sequence	1.9	up	biological process unknown	1.57562E-06
2	X74804	H.sapiens D54 mRNA	1.6	up	biological process unknown	0.003756293
2	X78712	H.sapiens mRNA for glycerol kinase testis specific 2	1.5	up	glycerol metabolism	0.000155077
3	AF009265	Homo sapiens clone FB2C5 Cri-du-chat region mRNA	1.4	down	biological process unknown	0.000445326
3	AF088018	Homo sapiens full length insert cDNA clone YY88A05	1.3	down	biological process unknown	4.72428E-05
3	AF131855	Homo sapiens clone 25056 mRNA sequence	1.5	down	biological process unknown	2.84387E-07
3	AF147343	Homo sapiens full length insert cDNA clone YB31B05	1.9	down	biological process unknown	5.47705E-06

3	AF147369	Homo sapiens full length insert cDNA clone YB64E02	1.4	down	biological process unknown	0.00341564
3	AK001228	Homo sapiens cDNA FLJ10366 fis, clone NT2RM2001420	1.8	down	biological process unknown	2.26217E-05
3	AK002109	Homo sapiens cDNA FLJ11247 fis, clone PLACE1008693	1.4	down	biological process unknown	0.001115375
3	AK002150	Homo sapiens cDNA FLJ11288 fis, clone PLACE1009607	1.8	down	biological process unknown	0.000234275
3	AK002173	Homo sapiens cDNA FLJ11311 fis, clone PLACE1010102	1.4	down	biological process unknown	0.006648993
3	AK022850	Homo sapiens cDNA FLJ12788 fis, clone NT2RP2001946	1.4	down	biological process unknown	1.53982E-07
3	AK023600	Homo sapiens cDNA FLJ13538 fis, clone PLACE1006617	1.5	down	biological process unknown	0.008859778
3	AK026099	Homo sapiens cDNA: FLJ22446 fis, clone HRC09457	2.2	down	biological process unknown	0.009163995
3	AK026571	Homo sapiens cDNA: FLJ22918 fis, clone KAT06627	1.4	down	biological process unknown	0.002059913
3	AL049342	Homo sapiens mRNA; cDNA DKFZp566A193 (from clone DKFZp566A193)	1.5	down	biological process unknown	0.000592814
3	AL109715	Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 430268	1.4	down	biological process unknown	0.000185708
3	AL109818	Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 191017	1.3	down	biological process unknown	0.000423743
3	AL137313	Homo sapiens mRNA; cDNA DKFZp761M10121 (from clone DKFZp761M10121)	1.4	down	biological process unknown	0.007227646
3	AL137703	Homo sapiens mRNA; cDNA DKFZp564P1772 (from clone DKFZp564P1772)	1.2	down	DNA packaging	0.002187628
3	AL162065	Homo sapiens mRNA; cDNA DKFZp762K135 (from clone DKFZp762K135)	1.5	down	transcription regulation	0.000696087
3	AL359056	Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 328974	1.3	down	biological process unknown	0.003624825
3	D28446	Human mRNA for cytokeratin 8, 5'UTR (sequence from the 5'cap to the start codon)	1.8	down	biological process unknown	0.004367359
3	L39924	Homo sapiens (clone HPL14E8) mRNA, partial EST	1.4	down	biological process unknown	2.23848E-05
3	NM_000143	Homo sapiens fumarate hydratase (FH) mRNA	1.8	down	fumarate metabolism	0.000064016
3	NM_002048	Homo sapiens growth arrest-specific 1 (GAS1) mRNA	1.7	down	cell cycle arrest	0.000240229
3	NM_002413	Homo sapiens microsomal glutathione S-transferase 2 (MGST2) mRNA, and translated products	1.7	down	leukotriene metabolism	0.000741744
3	NM_003705	Homo sapiens solute carrier family 25 (mitochondrial carrier, Aralar), member 12 (SLC25A12) mRNA	1.8	down	mitochondrial transport	4.58982E-09
3	NM_004837	Homo sapiens geranylgeranyl diphosphate synthase 1 (GGPS1), mRNA	1.4	down	lipid metabolism	0.001170041
3	NM_006550	Homo sapiens fibrinogen silencer binding protein (FSBP) mRNA	1.8	down	developmental processes	0.008701064
3	NM_014028	Homo sapiens HSPC019 protein (HSPC019), mRNA	1.2	down	biological process unknown	1.26357E-08
3	NM_014118	Homo sapiens PRO0159 protein (PRO0159), mRNA	1.9	down	biological process unknown	4.29652E-05
3	NM_014157	Homo sapiens HSPC065 protein (HSPC065), mRNA	1.4	down	biological process unknown	0.000726736
3	NM_018394	Homo sapiens hypothetical protein FLJ11342 (FLJ11342), mRNA	2	down	nitrogen metabolism	0.002850132
3	NM_019845	Homo sapiens candidate mediator of the p53-dependent G2 arrest (REPRIMO), mRNA	1.3	down	cell cycle arrest	0.005328642
3	U00960	Human clone KDBB1.2 (CAC)n/(GTG)n repeat-containing mRNA	1.6	down	biological process unknown	8.63988E-10
3	AB029396	Homo sapiens hu-GlcAT-P mRNA for glucuronyltransferase, complete cds	1.2	up	carbohydrate metabolism	0.005055305
3	AB037770	Homo sapiens mRNA for KIAA1349 protein, partial cds	1.3	up	transcription regulation	3.68379E-05
3	AB040672	Homo sapiens GalNAc-T9 mRNA for UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase, complete cds	1.1	up	carbohydrate metabolism	2.60519E-06
3	AF052115	Homo sapiens clone 23688 mRNA sequence	1.3	up	biological process unknown	0.000309023
3	AF086535	Homo sapiens full length insert cDNA clone ZE07G05	1.7	up	biological process unknown	5.46007E-05
3	AF088035	Homo sapiens full length insert cDNA clone ZC26G04	1.5	up	biological process unknown	0.001835077
3	AF090920	Homo sapiens clone HQ0352 PRO0352 mRNA, partial cds	1.2	up	biological process unknown	0.001037299
3	AF147349	Homo sapiens full length insert cDNA clone YB40C09	1.1	up	biological process unknown	6.10882E-05
3	AF320908	Homo sapiens MAGE-D4 (MAGED4) mRNA, partial cds	1.4	up	neurogenesis	1.9466E-11
3	AK023060	Homo sapiens cDNA FLJ12998 fis, clone NT2RP3000267	1.6	up	energy pathways	0.000269399
3	AK024244	Homo sapiens cDNA FLJ14182 fis, clone NT2RP2004675	2.1	up	biological process unknown	1.01781E-07
3	AK025686	Homo sapiens cDNA: FLJ22033 fis, clone HEP08810, highly similar to HSU43374 Human normal keratinocyte mRNA	1.6	up	biological process unknown	0.000105142
3	AL049679	Human gene from PAC 97K10, chromosome X, similar to heparan-sulphate 6-sulfotransferase	1.3	up	heparin sulfate biosynthesis	1.3547E-09
3	AL137519	Homo sapiens mRNA; cDNA DKFZp434A1518 (from clone DKFZp434A1518); partial cds	1.9	up	microtubule-based movement	0
3	D63487	Human mRNA for KIAA0153 gene, partial cds	1.2	up	cytoskeleton integrity	8E-15
3	L19778	Homo sapiens histone H2A.1b mRNA, complete cds	1.8	up	nucleosome assembly	8.01858E-06
3	L23544	Human (clone z159) retinal mRNA	1.1	up	biological process unknown	1.68491E-08
3	NM_001075	Homo sapiens UDP glycosyltransferase 2 family, polypeptide B10 (UGT2B10) mRNA	1.9	up	metabolism	1.01363E-05
3	NM_001718	Homo sapiens bone morphogenetic protein 6 (BMP6) mRNA	1.1	up	cell growth and maintenance	0.004084709
3	NM_002232	Homo sapiens potassium voltage-gated channel, shaker-related subfamily, member 3 (KCNA3) mRNA	1.5	up	potassium transport	0.00010528
3	NM_002416	Homo sapiens monokine induced by gamma interferon (MIG), mRNA	1.2	up	immune response	0.003447458
3	NM_006332	Homo sapiens interferon, gamma-inducible protein 30 (IFI30) mRNA	1.7	up	immune response	8.38588E-06
3	NM_006581	Homo sapiens fucosyltransferase 9 (alpha (1,3) fucosyltransferase) (FUT9) mRNA	1.2	up	carbohydrate metabolism	0.002088679
3	NM_006782	Homo sapiens zinc-finger protein in MEN1 region (MCG4), mRNA	1.6	up	cell growth and maintenance	0.000808155
3	NM_006925	Homo sapiens splicing factor, arginine/serine-rich 5 (SFRS5), mRNA	1.4	up	mRNA processing	4.88143E-05

3	NM_013363	Homo sapiens procollagen C-endopeptidase enhancer 2 (PCOLCE2), mRNA	1.5	up	cell growth and maintenance	0.00114394
3	NM_014470	Homo sapiens GTP-binding protein (RHO6), mRNA	1.7	up	cell growth and maintenance	0.001556582
3	NM_017721	Homo sapiens hypothetical protein FLJ20241 (FLJ20241), mRNA	1.5	up	cytoskeleton integrity	2.11999E-05
3	NM_017912	Homo sapiens hypothetical protein FLJ20637 (FLJ20637), mRNA	1.4	up	ubiquitination	4.50622E-06
3	NM_018224	Homo sapiens hypothetical protein FLJ10803 (FLJ10803), mRNA	1.6	up	biological process unknown	6.82061E-07
4	AF086084	Homo sapiens full length insert cDNA clone YZ84C01	1.3	down	biological process unknown	0.000937429
4	AK000165	Homo sapiens cDNA FLJ20158 fis, clone COL08935	1.2	down	biological process unknown	0.007709425
4	AK001125	Homo sapiens cDNA FLJ10263 fis, clone HEMBB1000991	1.3	down	biological process unknown	0.00420537
4	AK021722	Homo sapiens cDNA FLJ11660 fis, clone HEMBA1004610	1.5	down	biological process unknown	0.00033026
4	AK023224	Homo sapiens cDNA FLJ13162 fis, clone NT2RP3003625	1.4	down	biological process unknown	0.00060531
4	L07383	Homo sapiens E2 small nucleolar RNA	1.8	down	biological process unknown	0.000200539
4	NM_002133	Homo sapiens heme oxygenase (decycling) 1 (HMOX1), mRNA	2.8	down	C21-steroid hormone biosynthesis	1.74019E-07
4	NM_002563	Homo sapiens purinergic receptor P2Y, G-protein coupled, 1 (P2RY1) mRNA	1.2	down	G-protein signaling, activates PLC	0.001918707
4	NM_014664	Homo sapiens KIAA0615 gene product (KIAA0615), mRNA	1.6	down	proteolysis and peptidolysis	0.00172726
4	U00925	Human clone C4E 1.61 (CAC)n/(GTG)n repeat-containing mRNA	1.4	down	biological process unknown	2.82531E-06
4	U79249	Human clone 23839 mRNA sequence	2.2	down	biological process unknown	0.004528961
4	U80770	Human EST clone 251800 mariner transposon Hsmar1 sequence	1.7	down	chromatin binding	0.001639818
4	AF091033	Homo sapiens GTP-binding protein RAB19B (RAB19B) mRNA, partial cds	1.8	up	intracellular protein traffic	0.001388839
4	AK024067	Homo sapiens cDNA FLJ14005 fis, clone Y79AA1002361, similar to Rattus norvegicus mRNA for protein phosphatase 1	1.7	up	glycogen metabolism	2.59537E-10
4	AL137724	Homo sapiens mRNA; cDNA DKFZp434D1319 (from clone DKFZp434D1319); partial cds	1.3	up	cell growth and maintenance	0.000010435
4	NM_002871	Homo sapiens RAB interacting factor (RABIF) mRNA	1.5	up	non-selective vesicle transport	0.000179949
4	NM_004113	Homo sapiens fibroblast growth factor 12B (FGF12B) mRNA	1.6	up	signal transduction	0.004340852
4	NM_006765	Homo sapiens Putative prostate cancer tumor suppressor (N33), mRNA	1.1	up	protein-nucleus import	9.01884E-05
4	NM_012200	Homo sapiens beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase I) (B3GAT3), mRNA	1.5	up	carbohydrate metabolism	0.000264031
4	NM_016109	Homo sapiens PPAR(gamma) angiopoietin related protein (PGAR), mRNA	1.3	up	cell growth and maintenance	1.86227E-08
4	NM_017737	Homo sapiens hypothetical protein FLJ20275 (FLJ20275), mRNA	1.6	up	cell growth and maintenance	0.009220201
4	U79242	Human clone 23560 mRNA sequence	1.7	up	biological process unknown	0.000772738
4	U92017	Human clone 199288 defective mariner transposon Hsmar2 mRNA sequence	1.4	up	biological process unknown	9.44859E-07
5	AF084362	Homo sapiens lipoate-protein ligase B mRNA, partial cds	1.3	down	cell cycle	0.000224514
5	AF086220	Homo sapiens full length insert cDNA clone ZC66B10	1.6	down	biological process unknown	4.01705E-05
5	AF147395	Homo sapiens full length insert cDNA clone YI62C12	1.5	down	biological process unknown	0.001884459
5	AF207831	Homo sapiens guanine nucleotide exchange factor (ARHGEF6) mRNA, 5' UTR and partial cds	2.1	down	biological process unknown	1.18416E-09
5	AK026728	Homo sapiens cDNA: FLJ23075 fis, clone LNG05768	1.6	down	biological process unknown	0.001440961
5	AL050182	Homo sapiens mRNA; cDNA DKFZp586A1923 (from clone DKFZp586A1923)	1.9	down	microtubule-based process	0.004033682
5	AL137511	Homo sapiens mRNA; cDNA DKFZp761M222 (from clone DKFZp761M222)	1.3	down	electron transport	0.001218442
5	AL137631	Homo sapiens mRNA; cDNA DKFZp434B205 (from clone DKFZp434B205); partial cds	1.6	down	intracellular protein traffic	0.009583668
5	M11948	Human promyelocytic leukemia cell mRNA, clones pHH58 and pHH81	1.5	down	biological process unknown	0.000240291
5	NM_000016	H.sapiens acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain (ACADM), mRNA	2.3	down	fatty acid beta-oxidation	0.00124041
5	NM_000925	Homo sapiens pyruvate dehydrogenase (lipoamide) beta (PDHB) mRNA	1.7	down	glucose metabolism	0.000125179
5	NM_001232	Homo sapiens calsequestrin 2, cardiac muscle (CASQ2), mRNA	1.8	down	striated muscle contraction	3.21972E-10
5	NM_001359	Homo sapiens 2,4-dienoyl CoA reductase 1, mitochondrial (DECR1), mRNA	1.8	down	fatty acid beta-oxidation	0.001979939
5	NM_001995	Homo sapiens fatty-acid-Coenzyme A ligase, long-chain 1 (FACL1), nuclear gene encoding mitochondrial protein, mRNA	2	down	fatty acid metabolism	6E-15
5	NM_002079	Homo sapiens glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1) (GOT1) mRNA	1.7	down	aspartate catabolism	0.004712809
5	NM_002103	Homo sapiens glycogen synthase 1 (muscle) (GYS1) mRNA	1.7	down	glycogen metabolism	0.00081699
5	NM_002218	Homo sapiens inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein) (ITIH4) mRNA	1.2	down	proteolysis and peptidolysis	0.003586815
5	NM_002492	Homo sapiens NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5 (16kD, SGDH) (NDUFB5) mRNA	1.9	down	complex I (NADH to ubiquinone)	2.09703E-05
5	NM_002493	Homo sapiens NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6 (17kD, B17) (NDUFB6) mRNA	1.4	down	complex I (NADH to ubiquinone)	0.000392787
5	NM_002756	Homo sapiens mitogen-activated protein kinase kinase 3 (MAP2K3), mRNA	2	down	signal transduction	5.21806E-08
5	NM_003000	Homo sapiens succinate dehydrogenase complex, subunit B, iron sulfur (Ip) (SDHB), mRNA	2.4	down	electron transport	0.000318155
5	NM_003754	Homo sapiens eukaryotic translation initiation factor 3, subunit 5 (epsilon, 47kD) (EIF3S5) mRNA	1.8	down	translational regulation, initiation	0.004751168
5	NM_005006	H.sapiens NADH dehydrogenase (ubiquinone) Fe-S protein 1 (75kD) (NADH-coenzyme Q reductase) (NDUFS1) mRNA	2.4	down	complex I (NADH to ubiquinone)	0.001563059
5	NM_006759	Homo sapiens UDP-glucose pyrophosphorylase 2 (UGP2), mRNA	2	down	UDP-glucose metabolism	1.03084E-07
5	NM_007079	Homo sapiens protein tyrosine phosphatase type IVA, member 3 (PTP4A3), mRNA	1.7	down	DNA replication	1.15352E-05
5	NM_007103	Homo sapiens NADH dehydrogenase (ubiquinone) flavoprotein 1 (51kD) (NDUFV1), mRNA	1.9	down	energy pathways	0.000114478
5	NM_015831	Homo sapiens acetylcholinesterase (YT blood group) (ACHE), transcript variant E4-E5, mRNA	1.9	down	synaptic transmission	4.33511E-07

5	NM_018464	Homo sapiens uncharacterized hematopoietic stem/progenitor cells protein MDS029 (MDS029), mRNA	1.9	down	microtubule-based process	3.07654E-10
5	NM_018533	Homo sapiens hypothetical protein PRO2706 (PRO2706), mRNA	1.8	down	biological process unknown	9.05355E-07
5	S74678	heterogeneous nuclear ribonucleoprotein complex K [human, mRNA, 2302 nt]	2.2	down	RNA processing	2.462E-12
5	U00942	Human clone A9A2BR17 (CAC)n/(GTG)n repeat-containing mRNA	1.7	down	biological process unknown	0.00089146
5	U56725	Human heat shock protein mRNA, complete cds	1.4	down	spermatid development	0.000542513
5	X03168	Human mRNA for S-protein	1.9	down	cell adhesion	0.001129344
5	Y12235	H.sapiens mRNA for fructose-1,6-biphosphatase, alternative 5'UTR	1.4	down	biological process unknown	0.001089767
5	AJ001904	Homo sapiens cDNA selection product, DCR1-24.0	1.2	up	biological process unknown	0.001378771
5	AK022090	Homo sapiens cDNA FLJ12028 fis, clone HEMBB1001850	1.4	up	biological process unknown	0.002103195
5	AK022282	Homo sapiens cDNA FLJ12220 fis, clone MAMMA1001082	1.9	up	biological process unknown	1.02208E-08
5	AK022343	Homo sapiens cDNA FLJ12281 fis, clone MAMMA1001745	1.4	up	biological process unknown	0.002352396
5	AK024532	Homo sapiens cDNA: FLJ20879 fis, clone ADKA03124	1.1	up	biological process unknown	0.007801208
5	D16875	Human HepG2 3' region cDNA, clone hmd1f06	1.4	up	biological process unknown	0.000715273
5	NM_000596	Homo sapiens insulin-like growth factor binding protein 1 (IGFBP1), mRNA	1.9	up	signal transduction	2.1353E-11
5	NM_001647	Homo sapiens apolipoprotein D (APOD) mRNA	1.7	up	transport	0.002302229
5	NM_002612	Homo sapiens pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4) mRNA	1.7	up	glucose metabolism	0.000173786
5	NM_003492	Homo sapiens ITBA1 gene (ITBA1), mRNA	1.8	up	biological process unknown	0.000131711
5	NM_004566	Homo sapiens 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) mRNA	3.3	up	fructose metabolism	2E-14
5	NM_004906	Homo sapiens gene predicted from cDNA with a complete coding sequence (KIAA0105) mRNA	1.8	up	cell cycle	7.90062E-08
5	NM_005920	Homo sapiens MADS box transcription enhancer factor 2, polypeptide D (myocyte enhancer factor 2D) (MEF2D) mRNA	1.4	up	transcription regulation	0.009663229
5	NM_020162	Homo sapiens hypothetical protein DKFZp762F2011 (DKFZp762F2011), mRNA	2.1	up	developmental processes	6.83262E-07
5	NM_020193	Homo sapiens GL002 protein (GL002), mRNA	1.9	up	cytoskeleton integrity	5.20558E-05
5	U21072	Human tandem repeat region from facioscapulohumeral muscular dystrophy-associated mRNA clone cDNAdelta4	1.3	up	biological process unknown	1.35132E-06
5	U52832	Homo sapiens Cri-du-chat region mRNA, clone CSC3	1.6	up	biological process unknown	0.000895676

Appendix A.2 Microarray low laser hit list ($p < 0.01$). By microarray analysis, 298 genes were up- or downregulated after a HFD. Of those 298, 6 are known to be involved in oxidative phosphorylation or mitochondrial function.

Gene	Accession number	Forward primer	Probe	Reverse primer
PDK4	NM_002612	TTTCCAGACCAACCAATTCACA	AGCATATGATGGAGGTGAGAAGGAACATACACG	CGCATTGCATTCTTAAATAGTTCAA
PFKFB3	NM_004566	ACTACTAGAGAGAGGAGACACATGATCCT	AACGCCTTAAAGTCATTTTCTTTGGCAAAATG	CGCACACCGACTCGATGA
GYS1	NM_002103	GAGAACGCAGTGCTCTTCGAA	TGCCACCCACCTTGTTAGCCACCT	TCGTCTGCAGCACCGTGTAG
GALT	NM_000155	ACGCCGCAGCAGCAA	TGCTGATGGTCGTTTGCCCGG	CATCCTGCAGCGGGTTGTA
RPLP0	NM_001002	CCATTCTATCATCAACGGGTACAA	TCTCCACAGACAAGGCCAGGACTCG	AGCAAGTGGGAAGGTGTAATCC
PDHB	NM_000925	GAAGGAGGCTGGCCACAGT	Sybr Green I detection	TTGAACGCAGGACCTTCCAT
MAP2K3	NM_002756	CTACATGGCCCCTGAGAGGAT	Sybr Green I detection	TCCAGACG TCGGACTTGACA
UGP2	NM_006759	AATCAACCCACCCAATGGAA	Sybr Green I detection	GTGAGTGTCCCGCCCTTTAC
18S	NM_022551	GATATGCTCATGTGGTGTTGA	Sybr Green I detection	ACGTTCCACCTCATCCTCA
mmPDK4	NM_013743	TTCCATGAGAAGAGCCCAGAA	CCCTGTCAGAGTTTGTAGACACGCTGGTCA	GCCATTGTAGGGACCACATTATG
mmPFKFB3	NM_133232	TGCAGAGGAGATGCCATACCT	TCAGTTTCAGGACAGTGTGGAGCGGA	ACCCGGCACCCATATGC
mmGALT	NM_016658	GCCATGATGGGCTGTTCTAAC	TGCTAGCCCAAACCTGGCAGTGG	GGGCGATATCTGGCAGGAA
mmGYS1	NM_030678	CCGGCTTTGGCTGCTTTA	TAAATGCCGTAAGCTGAGGGATCTGCGAT	CGGAACCTCCGATCCAGAAT
mmCycB	NM_011149	GGTGGAGAGCACCAAGACAGA	ATCCTTCAGTGGCTTGTCCCGGCT	GCCGGAGTCGACAATGATG

Appendix A.3 Oligonucleotide sequences for primer/probe sets used for qRT-PCR. For all assays performed using Taqman primers and probe, RPLP0, which is the human equivalent of the murine gene 36B4, was used as the internal control. For all assays performed using SYBR Green I detection, 18S was used. Cyclophilin B was used as the internal control for all murine assays. PDK4, pyruvate dehydrogenase kinase, isoenzyme 4; PFKFB3, fructose-2,6-biphosphatase 3; GYS1, glycogen synthase 1 (muscle); GALT, galactose-1-phosphate uridyltransferase; RPLP0, ribosomal protein, large, P0; PDHB, pyruvate dehydrogenase (lipoamide) beta; MAP2K3, mitogen-activated protein kinase kinase 3; UGP2, UDP-glucose pyrophosphorylase 2; 18S, ribosomal protein S18; mm, mus musculus; mmPDK4, pyruvate dehydrogenase kinase, isoenzyme 4; mmPFKFB3, fructose-2,6-biphosphatase 3; mmGALT, galactose-1-phosphate uridylyltransferase; mmGYS1, glycogen synthase 1 (muscle); mmCycB, peptidylprolyl isomerase B (cyclophilin B).

CLUSTER	ACCESSION	GENE DESCRIPTION	FOLD CHANGE	DIRECTION	FUNCTION	BONFERONNI P-VALUE
1	AF075006	Homo sapiens full length insert cDNA YH98F07	2.2	down	biological process unknown	0.018577505
1	AJ227908	Homo sapiens partial mRNA; ID ED70-2A	2.9	down	biological process unknown	0.011717709
1	AK025950	Homo sapiens cDNA: FLJ22297 fis, clone HRC04521	1.8	down	cell cycle	0.048899404
1	AK026675	Homo sapiens cDNA: FLJ23022 fis, clone LNG01117	2.3	down	biological process unknown	0.035757495
1	AL359586	Homo sapiens mRNA; cDNA DKFZp762H185 (from clone DKFZp762H185)	1.8	down	biological process unknown	0.028866444
1	NM_004793	Homo sapiens protease, serine, 15 (PRSS15), mRNA	2.0	down	proteolysis and peptidolysis	0.025259937
1	NM_006335	Homo sapiens translocase of inner mitochondrial membrane 17 (yeast) homolog A (TIM17), mRNA	2.0	down	mitochondrial translocation	0.017267625
1	X74606	H.sapiens XAP-1 mRNA	1.6	down	biological process unknown	0.014891916
1	AK000689	Homo sapiens cDNA FLJ20682 fis, clone KAIA3543, highly similar to AF131826 H.sapiens clone 24945 mRNA	1.7	up	transcription	0.035494872
1	AK021789	Homo sapiens cDNA FLJ11727 fis, clone HEMBA1005374	2.3	up	biological process unknown	0.021014007
1	AK021815	Homo sapiens cDNA FLJ11753 fis, clone HEMBA1005583	1.6	up	glycogen biosynthesis	0.029970463
1	AK026764	Homo sapiens cDNA: FLJ23111 fis, clone LNG07835	2.2	up	biological process unknown	0.017174109
1	NM_002319	Homo sapiens leucine-rich neuronal protein (LRN) mRNA, complete sequence	2.1	up	biological process unknown	0.036158241
1	NM_006145	Homo sapiens heat shock 40kD protein 1 (HSPF1) mRNA	1.8	up	protein folding	0.024477331
1	X98115	H.sapiens mRNA for cardiac titin, clone ZisL	2.2	up	cytoskeletal anchoring	0.014638631
2	AB031038	Homo sapiens mRNA for hTbr2, complete cds	2.2	down	embryogenesis / morphogenesis	0.046379407
2	AF086452	Homo sapiens full length insert cDNA clone ZD83B06	2.6	down	biological process unknown	0.017967876
2	AK023814	Homo sapiens cDNA FLJ13752 fis, clone PLACE3000352	1.6	down	biological process unknown	0.046664747
2	NM_001486	Homo sapiens glucokinase (hexokinase 4) regulatory protein (GCKR) mRNA	1.8	down	nitrogen metabolism	0.012285037
2	NM_005691	Homo sapiens ATP-binding cassette, sub-family C (CFTR/MRP), member 9 (ABCC9), mRNA	1.9	down	biological process unknown	0.014468696
2	NM_006033	Homo sapiens lipase, endothelial (LIPG), mRNA	1.5	down	lipid metabolism	0.014177713
2	NM_013356	Homo sapiens monocarboxylate transporter 3 (SLC16A8), mRNA	1.6	down	transport	0.049486595
2	NM_014632	Homo sapiens KIAA0750 gene product (KIAA0750), mRNA	3.4	down	cell motility	0.010994883
2	NM_018366	Homo sapiens hypothetical protein FLJ11230 (FLJ11230), mRNA	1.6	down	biological process unknown	0.014768323
2	AF009290	Homo sapiens clone HEC5 Cri-du-chat region mRNA	1.3	up	biological process unknown	0.029964519
2	AF097645	Homo sapiens candidate tumor suppressor protein DICE1 mRNA, complete cds	1.5	up	cell growth and/or maintenance	0.011826773
2	AK026339	Homo sapiens cDNA: FLJ22686 fis, clone HSI10987	1.5	up	cytoskeleton organization / biogenesis	0.011832297
2	AL050125	Homo sapiens mRNA; cDNA DKFZp586F071 (from clone DKFZp586F071)	1.6	up	biological process unknown	0.048827711
2	AL353951	Homo sapiens mRNA; cDNA DKFZp761A0423 (from clone DKFZp761A0423)	1.2	up	biological process unknown	0.020453577
2	M33197	Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, complete cds	1.6	up	biological process unknown	0.02396184
2	NM_003948	Homo sapiens cyclin-dependent kinase-like 2 (CDC2-related kinase) (CDKL2), mRNA	1.8	up	biological process unknown	0.0294794
2	NM_004293	Homo sapiens guanine deaminase (GDA), mRNA	1.7	up	nucleic acid metabolism	0.029360338
2	NM_006249	Homo sapiens proline-rich protein BstNI subfamily 3 (PRB3), mRNA	1.7	up	cytoskeleton organization / biogenesis	0.015708017
2	NM_006268	Homo sapiens requiem, apoptosis response zinc finger gene (REQ) mRNA	1.4	up	transcription regulation	0.032503592
2	NM_014684	Homo sapiens KIAA0373 gene product (KIAA0373), mRNA	1.8	up	muscle contraction	0.042243882
2	NM_016098	Homo sapiens HSPC040 protein (LOC51660), mRNA	1.5	up	porphyrin biosynthesis	0.016111001
2	NM_016616	Homo sapiens NM23-H8 (LOC51314), mRNA	1.7	up	nucleic acid metabolism	0.024200036
3	AF056418	Homo sapiens clone FBA4 Cri-du-chat critical region mRNA	1.5	down	biological process unknown	0.031744664
3	AF131745	Homo sapiens clone 25110 mRNA sequence	1.9	down	ubiquinone biosynthesis	0.013476411
3	AF147315	Homo sapiens full length insert cDNA clone YA85A01	1.3	down	biological process unknown	0.010894018
3	AK021970	Homo sapiens cDNA FLJ11908 fis, clone HEMBB1000089	2.0	down	biological process unknown	0.041219865
3	L07033	Human hydroxymethylglutaryl-CoA lyase mRNA, complete cds	1.7	down	leucine metabolism	0.037290172
3	NM_000329	Homo sapiens retinal pigment epithelium-specific protein (65kD) (RPE65) mRNA	1.5	down	vision	0.044554398
3	NM_004045	Homo sapiens ATX1 (antioxidant protein 1, yeast) homolog 1 (ATOX1), mRNA	1.5	down	copper ion transport	0.014124624
3	NM_014425	Homo sapiens inversin (INVS), mRNA	1.8	down	vesicle transport	0.027775891
3	NM_015044	Homo sapiens KIAA1080 protein; Golgi-associated, gamma-adaptin ear containing, ARF-binding protein 2, mRNA	1.5	down	intracellular protein traffic	0.032004408
3	NM_018077	Homo sapiens hypothetical protein FLJ10377 (FLJ10377), mRNA	1.4	down	RNA processing	0.012490038

3	NM_018139	Homo sapiens hypothetical protein FLJ10563 (FLJ10563), mRNA	1.8	down	biological process unknown	0.042805641
3	AB046836	Homo sapiens mRNA for KIAA1616 protein, partial cds	1.4	up	cell growth and/or maintenance	0.014551636
3	M37190	Human ras inhibitor mRNA, 3' end	1.5	up	cell growth and/or maintenance	0.021971274
3	X66087	H.sapiens a-myb mRNA	1.6	up	transcription regulation	0.017827478
4	AK000257	Homo sapiens cDNA FLJ20250 fis, clone COLF6635	1.6	down	biological process unknown	0.023477455
4	AL117636	Homo sapiens mRNA; cDNA DKFZp434H205 (from clone DKFZp434H205)	1.4	down	biological process unknown	0.013849179
4	AL359559	Homo sapiens mRNA; cDNA DKFZp762O2215 (from clone DKFZp762O2215)	1.6	down	biological process unknown	0.012576309
4	AL390145	Homo sapiens mRNA; cDNA DKFZp762C115 (from clone DKFZp762C115)	1.6	down	biological process unknown	0.047102089
4	NM_020154	Homo sapiens chromosome 11 hypothetical protein ORF3 (LOC56851), mRNA	1.7	down	cell motility	0.040716234
4	X97303	H.sapiens mRNA for Ptg-12 protein	2.1	down	transcription regulation	0.026556997
4	AF085932	Homo sapiens full length insert cDNA clone YR56G07	1.6	up	biological process unknown	0.014081919
4	NM_004590	Homo sapiens small inducible cytokine subfamily A (Cys-Cys), member 16 (SCYA16), mRNA	1.5	up	immune response	0.011291698
4	NM_018247	Homo sapiens hypothetical protein FLJ10856 (FLJ10856), mRNA	1.5	up	mitochondrial processing	0.024924348
5	AF085899	Homo sapiens full length insert cDNA clone YQ15A02	1.6	down	biological process unknown	0.027363645
5	AF104921	Homo sapiens succinyl-CoA synthetase alpha subunit (SUCLA1) mRNA, complete cds	2.1	down	succinyl-CoA metabolism	0.028777168
5	AK000903	Homo sapiens cDNA FLJ10041 fis, clone HEMBA1001022	1.6	down	biological process unknown	0.031533837
5	NM_001151	Homo sapiens solute carrier family 25, member 4 (SLC25A4), nuclear gene encoding mitochondrial protein, mRNA	2.4	down	energy pathways	0.035615153
5	NM_012409	Homo sapiens prion gene complex, downstream (PRND), mRNA	1.9	down	biological process unknown	0.033394452
5	NM_012447	Homo sapiens stromal antigen 3 (STAG3), mRNA	1.8	down	male meiosis sister chromatid cohesion	0.029586542
5	NM_016497	Homo sapiens hypothetical protein (LOC51258), mRNA	1.9	down	biological process unknown	0.04049059
5	AK022226	Homo sapiens cDNA FLJ12164 fis, clone MAMMA1000605	2.5	up	biological process unknown	0.036196808
5	AL080113	Homo sapiens mRNA; cDNA DKFZp586K2322 (from clone DKFZp586K2322)	1.7	up	biological process unknown	0.010343509
5	NM_004887	Homo sapiens CXC chemokine in breast and kidney (BRAK) mRNA	1.5	up	immune response	0.011983042
5	NM_014012	Homo sapiens REM protein (REM), mRNA	1.2	up	cell surface receptor signal transduction	0.035096741
5	NM_014827	Homo sapiens KIAA0663 gene product (KIAA0663), mRNA	1.8	up	cytoskeleton organization / biogenesis	0.013963907

Appendix A.4 Microarray low laser hit list ($p < 0.05$). By microarray analysis, 72 genes were up- or downregulated after a HF/LCD. Of those 72, 7 are known to be involved in glucose metabolism.

Official Gene Symbol	Common Gene Name	Accession number	Forward primer	Probe	Reverse primer
PPARGC1A	PGC1α	NM_0013261	AGGTGAAAGTGTAATACTGTTGGTTGA	TGCTGAAGAGGGAAAGTGAGCGATTAGTTGA	CATGTAGAATTGGCAGGTGGAA
ND1	mtDNA	AF346985	CCCTAAAACCCGCCACATCT	CCATCACCCTCTACATCACCGCCC	GAGCGATGGTGAGAGCTAAGGT
genomicLPL		NC_000008	CGAGTCGTCTTTCTCCTGATGAT	ACATTCACCAGAGGGTC	TTCTGGATTCCAATGCTTCGA
CD36	CD36	NM_000072	AGTCACTGCGACATGATTAATGGT	CAGATGCAGCCTCATTTCCACCTTTTG	CTGCAATACCTGGCTTTTCTC
LPL	LPL	NM_000237	TATCCGCGTGATTGCAGAGA	CTAGCTGGTCCACATCTCCAAGTCCT	AGAGAGTCGATGAAGAGATGAATGG
FAS	FAS	NM_000043	TATGCTTCTTCGTGCAGCAGTT	AGCGCCTCCAGCACCCTGTTGT	GCTGCCACACGCTCCTCTAG
PCK1	PCK1	NM_002591	CAGGCGGCTGAAGAAGTATGA	AACTGCTGGTTGGCTCTCACTGACCC	AACCGTCTTGCTTTCGATCCT
SCD	SCD1	NM_005063	TGGCATTCCAGAATGATGTCTATG	CGTGACCACCGTGCCCACCACA	GGAATTATGAGGATCAGCATGTGT
ACADM	MCAD	NM_000016	TGCCCTGGAAAGGAAAACTTT	TGTAGAGCACCAAGCAATATCATTTATG	GTTCAACTTTCATTGCCATTTCAG
PPARA	PPARα	NM_001001928	GCTTTGGCTTTACGGAATACCA	AGCCATCTGAGCCAGGACAGCTTCCTAA	TGAAAGCGTGTCCGTGATGA
PLIN	perilipin	NM_002666	CACCGTGGCCATGTGGAT	CCCCTGAGCAGCCTGGCCC	GCCTGCATGGCCACTGAG
SORBS1	CAP	NM_001034954	CAAATTCCCTGAACTTCCTGAAA	CCAGCAAACTTCCGAAGAGGACAATCC	AGGAAACTGGTAGGTGGGAGTGTA
PNPLA2	ATGL	NM_020376	CCACGGCGCTGGTCA	TTGGCACCAGCCTCACCCAGG	GGGCCTCTTTAGATACCTCAATGA
LIPE	HSL	NM_005357	GACTTCCTCCGGGAGTATGTC	TGCATAAGGGATGCTTCTATGGCC	GCGTGAACTGGAAGCCCA
PPARG	PPARγ1	NM_005037	GTCAAACGAGAGTCAGCCTTTAACG	AGAGATGCCATTCTGGCCCACCAACTT	CCACGGAGCTGATCCCAA
PPARG	PPARγ2	NM_015869	GATACACTGTCTGCAAACATATCACAA	AGAGATGCCATTCTGGCCCACCAACTT	CCACGGAGCTGATCCCAA
CD68	CD68	NM_001040059	GCTTCTCTCATTCCCCTATGGA	CAGCTTTGGATTCATGCAGGACCTCC	ATGTAGCTCAGGTAGACAACCTTCTG
CD163	MAC-2	NM_004244	TGCAGAAAACCCCACAAAAAG	CACAACAGGTCGCTCATCCCGTCA	CAAGGATCCCGACTGCAATAA
CCL2	MCP-1	NM_002982	GATCTCAGTGCAGAGGCTCG	AGCTATAGAAGAATCACCAGCAGCAAGTGTCCC	AATGGTCTTGAAGATCACAGCTTCT
CCL3	MIP-1α	NM_002983	ACAGAATTTCATAGCTGACTACTTTGAGA	AGTGCTCCAAGCCCGGTGTCATCTTC	GCCGGCTTCGCTTGGT
RPS18	18S	NM_022551	CGCCGCTAGAGGTGAAATTC	ACCGGCGCAAGACGGACCAGA	CATTCTTGGCAAATGCTTTCG

Appendix A.5 Oligonucleotide sequences for primer/probe sets used for qRT-PCR. PGC1α, peroxisome proliferative activated receptor, gamma, coactivator 1, alpha; ND1, NADH dehydrogenase, subunit 1; genomicLPL, genomic lipoprotein lipase; CD36, CD36 molecule (thrombospondin receptor); LPL, lipoprotein lipase; FAS, fatty acid synthase; PCK1, phosphoenolpyruvate carboxykinase 1; SCD1, stearoyl-CoA desaturase; MCAD, acyl-Coenzyme A dehydrogenase C-4 to C-12 straight chain; PPARα, peroxisome proliferative activated receptor, alpha; perilipin, lipid droplet associated protein; CAP, cbl-associated protein; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; PPARγ1, peroxisome proliferative activated receptor, gamma 1; PPARγ2, peroxisome proliferative activated receptor, gamma 2; CD68, CD68 antigen; MAC-2, macrophage-associated antigen; MCP-1, monocyte chemoattractant protein-1; MIP-1α, macrophage inflammatory protein 1, alpha subunit;18S, ribosomal protein S18. For all assays performed using Taqman primers and probe, 18S was used as the internal control.

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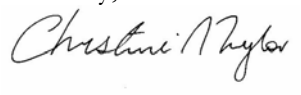

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